SECONDARY METABOLITES FROM XYLARIA ENDOPHYTES

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SECONDARY METABOLITES FROM XYLARIA ENDOPHYTES

The isolation and structure elucidation of secondary metabolites from *Xylaria* endophytes by chemical and spectroscopic methods

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To my beloved parents

and

my dear family: Maiya,

Awa, Rawya, Alla and Aseel

Abstract

Keywords: endophyte, fungi, NMR, secondary metabolites, structure elucidation, xylaria.

This thesis describes the isolation and structure elucidation of secondary metabolites from a number of endophytic *Xylaria* fungi. Six *Xylaria* endophytes were surface cultured on an aqueous malt extract-glucose medium. The fungus A311R, from a palm tree in Thailand, produced nonane-1,2,3-tricarboxylic acid, which was isolated for the first time as a natural product. Also isolated from the same fungus was spiculisporic acid; the first instance of isolation from a *Xylaria* fungus. The fungus 6RD12 produced cycloepoxydon, which was isolated for the first time from a *Xylaria* fungus, and 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one, which is a novel compound. The fungi A217R and A517R produced cytochalasin D, (S)-mellein and (3S,4S)-4-hydroxymellein as main secondary metabolites suggesting that the two fungi are the same species. The fungus X04 (*Xylaria* cf. *juruensis*) produced 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione as a novel compound, coriloxin as the main secondary metabolite in addition to (R)-mellein and a mixture of two stereoisomers of the 4-Hydroxymellein. The fungus 6RD8 produced (S)-*O*-methylmellein as the main secondary metabolite.

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Abbreviations

°C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
[α]	Specific rotatory power
δ	NMR chemical shift [ppm]
ν	Wave number [cm-1]
br	Broad
c	Concentration [g/100 mL]
cm	10^{-2} metre
COSY	Correlated Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement by Polarisation Transfer
e.g.	Example given
EI	Electron Ionisation
ES	Electrospray
g	Gram
H2BC	Heteronuclear 2-Bond Correlation
HMBC	Hetero nuclear Multiple Bond Correlation
HMQC	Hetero nuclear Multiple Quantum Coherence
HRMS	High Resolution Mass Spectrometry
Hz	Hertz
IR	Infrared
J	Spin-spin coupling constant [Hz]
L	Litre
Lit	Literature
m	Multiplet (in connection with NMR data)
MeOH	Methanol
MHz	Megahertz
mp	Melting point
MS	Mass Spectrometry
m/z	Molecular Ion
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
PLC	Preparative Layer Chromatography
ppm	parts per million
q	Quartet
RP	Reversed Phase
s	Singlet
sp.	Species
t	Triplet
TLC	Thin Layer Chromatography
UV	Ultra Violet
XRD	X-ray diffraction

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Chapter 1 Introduction

1.1 The Family Xylariaceae

Xylariaceae is a family of fungi belonging to the Ascomycota which traditionally has been divided into three main subclasses, plectomycetes, discomycetes and pyrenomycetes. The Xylariaceae belongs to the pyrenomycetes subclass. This family comprises of at least 40 genera [1-3] but nearly70 genera have now been listed [4]. The main habitat of the majority of the genera is wood [3] and most xylariaceous fungi are saprophytic and are found in decomposing wood, litter, fruits, seeds and leaves[5-7]. Additionally, many studies have shown that members of the Xylariaceae occur as endophytes [8-10]; living inside healthy plant tissues without causing any external disease symptoms [11-12]. Endophytic fungi have been shown to be promising producers of valuable products for the pharmaceutical and agricultural industries [13].

In 1979 using morphological data, Rogers [5] proposed a central core of genera for the family *Xylariaceae* comprising: *Hypoxylon*, *Rosellinia*, *Poronia*, *Podosordaria*, *Hypocopra*, *Daldinia*, *Nummalaria*, *Kretzschmaria*, *Camillea* and *Penzigia*.

In 1995 Whalley [14] proposed a scheme illustrating the relationships between these genera (Figure 1). The taxonomic classification of fungi species has been attempted in different ways. This includes use of:

- mycological data [15-16,]
- chemical data [17-18,]

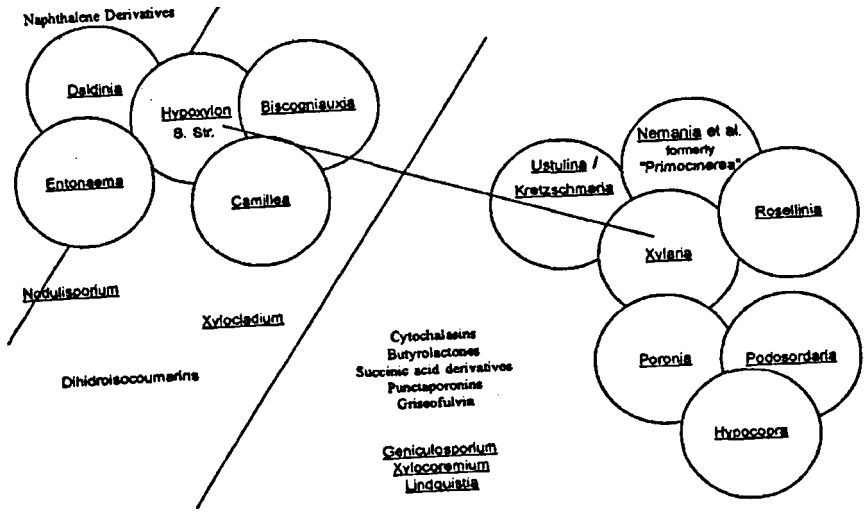


Figure 1 Scheme illustrating the relationships between *Xylariaceae* genera.

1.2 The Genus *Xylaria*

Xylaria is the oldest known genus of the family Xylariaceae. They are widespread around the world [19] and more than 500 species are believed to belong to this genus (Rogers. pers comm.). The fact that Xylaria species vary in shape, colour and size and the fact that they can appear to be different during different stages of maturation, has resulted in their classification being confused and as a result one species can have more than one name. Stromatal morphology was used in early research as the main basis for classification of Xylaria species [20-21]. Several attempts have been made to classify Xylaria and the most reliable one was derived in 1985 by Rogers [22-23] (Table 1), who used in place of the morphology of stromata (teleomorph) in the life cycle the morphology of the conidia (anamorph) produced by the fungi as main characters for classification.

Although this system is more satisfactory, it is incomplete, and many gaps need to be filled because not all the species have been cultured and few Xylaria species have anamorphic data. Until now no stable classification system is available for Xylaria

genera. The use of chemotaxonomy could help to highlight the relations between the different species [24].

Table 1 Classification of Xylaria genera according to Rogers [22-23]

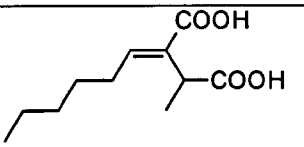
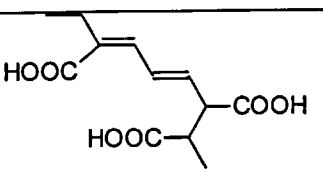
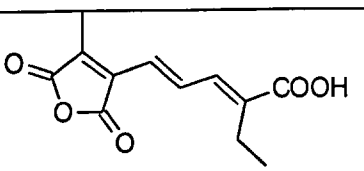
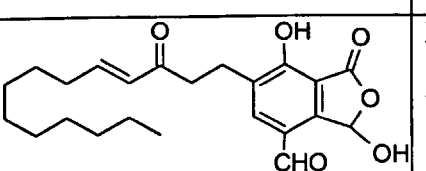
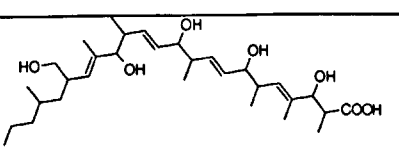
Section	Characteristic features
1	Conidia produced from palisade of conidiogenous cells over active young telemorphic stromata or at least not limited to specialised appendages, Conidiogenesis holoblastic. Conidia produced in more or less sympodial sequence, seeding individually and passively. This section contains 5 groups: *Xylaria polymorpha*, *X. hypoxylon*, *X. multiplex*, *X. pyramidata* and *X. pedunculata*.
2	Conidia produced on special and localised peg or hairy-shaped appendages on young teleomorphic stromata, Conidia apparently seeding individually and passively. This section is represented by the *X. comosa* group.
3	Conidia produced on special anamorphic stromata or conidiomata which usually are produced earlier in the year than telemorphic stromata. Telemorphic stromata never bear conidia, Conidiogenesis holoblastic. Conidia produced in more or less sympodial sequence, seeding individually and passively.e.g *X. cubensis*.
4	Conidia produced on young telemorphic stromata. Conidiogenesis apparently holoblastic. Conidia produced in tendem seeding forcibly. This section is represented by *X. furcata*.

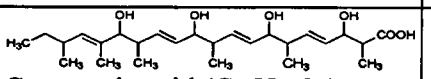
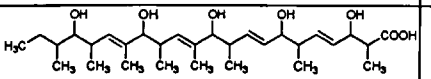
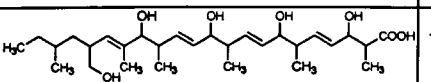
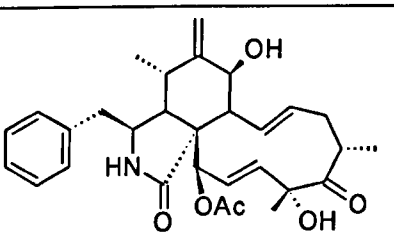
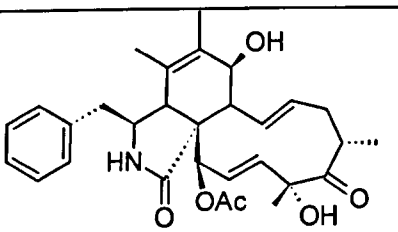
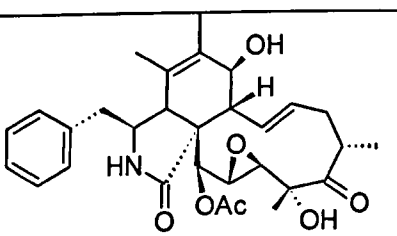
1.3 Secondary metabolite profile

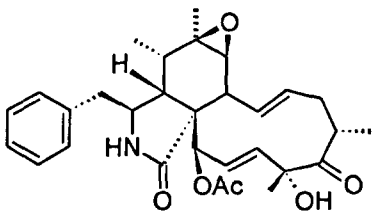
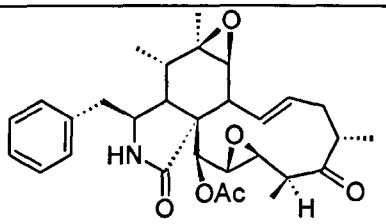
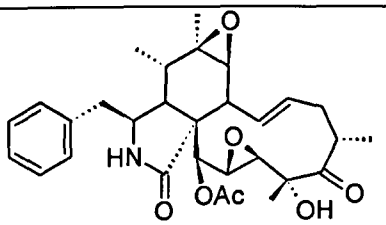
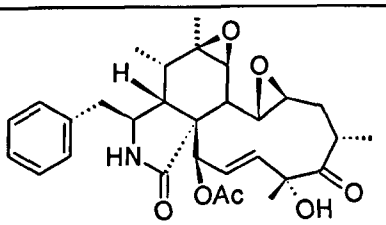
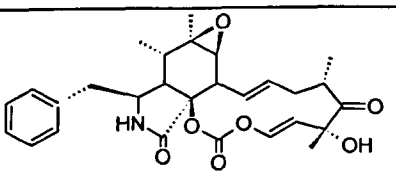
A fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus, an order, or even phylum, and has high differentiation power [18]. Secondary metabolites can only be used as taxonomic markers with good

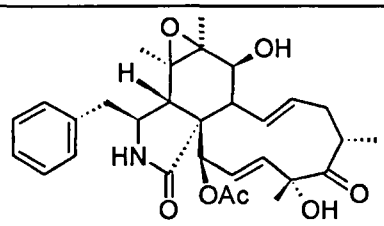
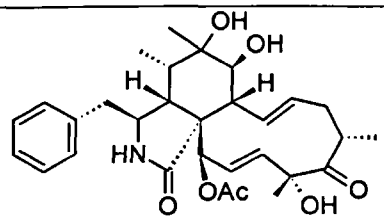
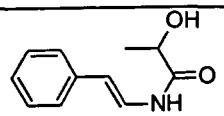
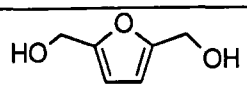
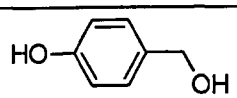
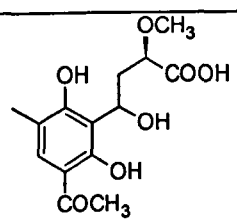
producer fungi, which can be grown under controlled laboratory conditions. Over 40 years of study have shown the fungi secondary metabolites are reliable and valuable taxonomy markers [14, 25]. Frisvad [26] in 1990 demonstrated that secondary metabolite profiles of *Talaromyces* were specific for each species and provided means of simple differentiation of taxa and highlighted their potential in ascomycete taxonomy. It is not unusual that different fungal species have one or more secondary metabolites in common. Cytochalasin D is one such metabolite. It is produced by several fungal species [27]. The major secondary metabolites produced by different fungal species in aqueous culture can be divided into a number of chemical groups including, dihydroisocoumarins and related compounds, sesquiterpene alcohols, cytochalasins and succinic acid derivatives [28]. The whole range of secondary metabolites, that have been isolated from various Xylaria species are illustrated in Table 2.

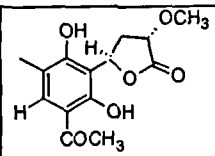
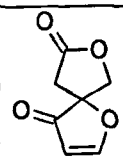
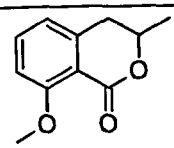
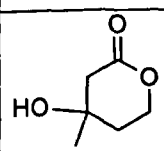
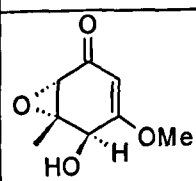
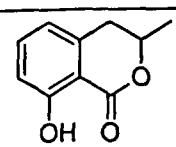
Table 2 Secondary metabolites from Xylaria species

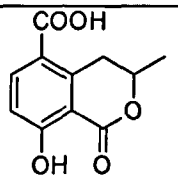
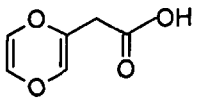
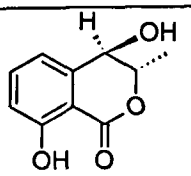
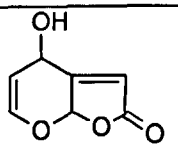
N	Compound structure	Xylaria species	Other species
1	 (E)-2-Hexylidene-3-methylsuccinic acid	X. polymorpha [29] X. mali [29] X. hypoxylon [29] X. longipes [30] X. multiplex [30] X. myosurus [31]	H. deustum P. pileiformis [29]
2	 Telfairic acid	X. telfairi [32]	
3	 2,3-Didehydrotelfairic Anhydride	X. telfairi [32]	
4	 Xylaral	X. polymorpha [33]	
5		X. cubensis [34]	

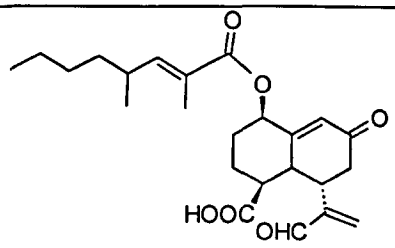
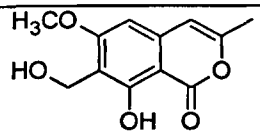
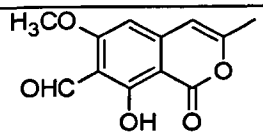
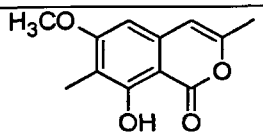
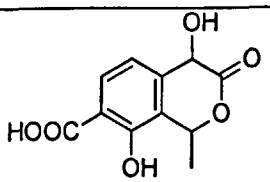
	Cubensic acid ($C_{30}H_{53}O_7$)		
6	 Cameronic acid ($C_{26}H_{44}O_6$)	*Xylaria* from Cameroon [35]	
7	 Berteric acid ($C_{30}H_{52}O_7$)	*X.* berterii [35]	
8	 Malaysic acid ($C_{29}H_{50}O_7$)	*Xylaria* species Malaysia [35]	
10	 Cytochalasin D	X. cubensis [34] X. species [36]	H. terricola [37]
11	 Cytochalasin C	X. species [36]	H. terricola [37]
12	 19,20-epoxycytochalasin C	X. obovata [38] X. hypoxylon [39]	

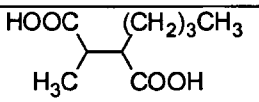
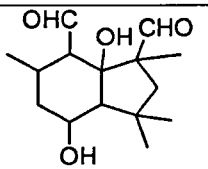
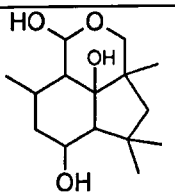
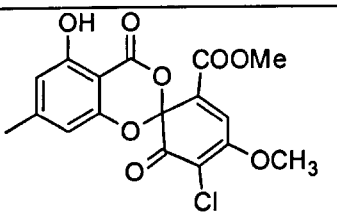
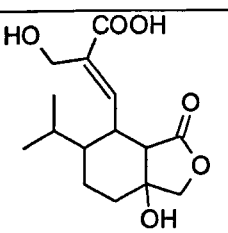
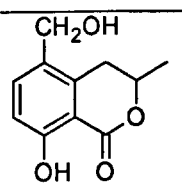
13	 Cytochalasin Q	X. obovata [38] X. hypoxylon [39]	
14	 Xylobovatin	X. obovata [38]	
15	 19,20-epoxycytochalasin Q	X. hypoxylon [39]	
16	 Cytochalasin R	X. hypoxylon [39]	
17	 Cytochalasin E		Rosellinia necatrix[40]

18	 Cytochalasin N	X. species [36]	Hypoxylon terricola [41]
19	 Cytochalasin O	Xylaria species [36]	Hypoxylon terricola [41]
20	 Xylaramide	X. longipes [42]	
21	 2,5-di(hydroxymethyl)furan	X. longipes [42]	Phellinus linteus [42]
22	 Tyrosol	X. longipes [42]	
23	 Globoscinic acid	X. globosa [43] X. obovata [43]	

24	 Globoscin	X. globosa [43] X. obovata [43]	
25	 Longianone	X. longiana [31]	
26	 S-8-O-methylmellein	X. species [36]	
27	 Mevalonolactone	X. species [36]	
28	 Coriloxin	X. species [36] X. obovata [43] X. badia [44]	Coriolus vernicipes [45]
29	 Mellein	(S) isomer : X. grammica [36] (R) isomer : X. longiana [31] X. badia [44]	Hypoxylon howieanum [46] Aspergillus oniki [47] Aspergillus

		X. species [36]	melleus [48]
30	 5-carboxymellein	X. species [36] X. myosurus [31]	Hypoxylon illitum, Hypoxylon mammatum [46]
31	 Xylaric acid	X. species [49]	
32	 4-hydroxymellein	X. grammica [36] X. longiana [19]	Aspergillus oniki [47] Aspergillus ochraceus [50] Aspergillus melleus [48, 32]
33	 Grammicin	X. grammica [36]	

34	 Feejeensci acid	X. feejeensis [36]	
35	 8-hydroxy-7-hydroxymethyl-6-methoxy-3-methylisocoumarin	X. species [36]	
36	 7-formyl-8-hydroxy-6-methoxy-3-methylisocoumarin	X. species [36]	
37	 3,7-dimethyl-8-hydrxy-6-methoxyisocoumarin	X. species [36]	
38	 4,8-dihydroxy-1-methyl-3-oxo-isochroman-7-carboxylic acid	X. species [36]	

39	 2-n-butyl-3- methylsuccinic acid	X. species [36]	
40	 Deacetylbotrydial	X. species [36]	
41	 Deacetyldihydrobotrydial	X. species [36]	
42	 Maldoxin	X. species [32]	
43	 Myosuric acid	X. myosurus [31]	
44	 5-hydroxymethylmellein	X. myosurus [31]	Hypoxylon illitum, [46] Valsa ceratosperma [51]

1.4 Endophytes

Endophyte (Gr. endon, within; phyton, plant) the term was first coined by de Bary in 1866 and has become deeply embedded in the literature ever since [52-53]. Stone *et al* defined endophytic organisms as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects [54]. First reports describing endophytes date back to the beginning of the twentieth century [55].

Fungi and bacteria are the most common microbes that can exist as endophytes. But the most frequently isolated endophytes are the fungi [56]. Examination of plant material can lead to the discovery of several endophytic fungi and bacteria. Many fungi would be specific to that particular host [57]. So it is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant can be host to one or more endophytes.

The number of endophytic species potentially associated to a plant species can reach several hundred [58].

Furthermore, the colonization of endophytes in marine algae [59] mosses and ferns [60-61] has also been reported. Only a few of these plants have been completely studied relative to their endophytic biology [56]. It is believed that up to 99% of microorganisms have yet to be discovered [62].

Consequently, environmental conditions such as temperature, soil and humidity, are considered to affect the nature and the number of endophytes [63].

Many scientists believe that plants growing in tropical rainforests, where competition for light and nutrients is fierce, are mostly host for bigger numbers of bioactive endophytes. Researchers recently noted that endophytes from tropical regions produced significantly more bioactive secondary metabolites than those from temperate parts of the world [64-66].

The question of how microbial endophytes get into their host plants is still not clear. Obviously most microbial fungal endophytes can gain access through the roots, but bacterial endophytes are not thought to invade plant tissue directly; instead, they tend to enter the plant through natural openings or wounds [67]. Endophytes may also infect plants by means of horizontal transmission, when their inocula are transported to another plant, or vertically when they infect the seed progeny of an infected plant [68].

1.5 Endophytes and their host relationship

Endophytes that colonize the interior of plant tissues usually get nutrition and protection from the host plant. In return, they grant profoundly enhanced health to the host plants by producing certain functional metabolites [69].

Scientific observations show that endophyte-infected plants often grow faster than non-infected ones [70-71]. It thought that the endophytes improve the hosts intake of nutritional elements such as nitrogen [72] and phosphorus [73-75]

Additionally, some endophytes are able to support the host plant with defence against some nematodes [76-77] and insect herbivores [78-80] as well as bacterial and fungal pathogens.[81-82].

1.6 Natural products and drug discovery

The fact that the human population is always exposed to new diseases such as AIDS and severe acute respiratory syndrome requires the discovery and development of new drugs to fight them.

Also more drugs are needed to efficiently treat parasitic protozoan and nematodal infections, such as malaria, leishmaniasis, trypanomiasis, and filariasis [56]. Malaria alone causes more deaths each year than any other single infectious disease with the exception of the AIDS virus and *Mycobacterium tuberculosis* [83].

Today natural product research still has massive impact on modern medicine development in that 49% of the new chemical products registered by the U.S. Food and Drug Administration are natural products or derivatives [84].

More than 20 natural product derived drugs were launched onto the global market between 2001 and 2005 and have undergone various stages of clinical development in all major therapeutic fields [85-86].

Over, 3,000 years ago, the Mayans used fungi grown on roasted green corn to treat intestinal ailments [87], whilst the Benedictine monks (800 AD) used *Papaver somniferum* as an anaesthetic and pain reliever [88].

The existence of endophytes has been known for over 100 years[55, 69]. It was however only during the later decades that endophytes have been investigated for their potential as sources of effective new drugs. Nowadays, endophytes are known to provide many modern medicines such as penicillin from the fungus *Penicillium notatum*, and bacitracin from the bacterium *Bacillus subtilis*. In addition, scientific researchers show that some plants which produce bioactive natural products have associated endophytes that produce the same natural products. This can lead to a more economical production line to an expensive drugs and products and effectively reducing its market price[56]. For instance paclitaxel, a highly functionalized diterpenoid and important anticancer agent that is found in the world's yew tree (*Taxus* spp.) [89], has been found to be produced by a novel paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* [90]. This raised a concept of genetic recombination of the endophyte with the host that can occurs during evolutionary time [69].

1.7 Natural products from endophytic fungi

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals [56]

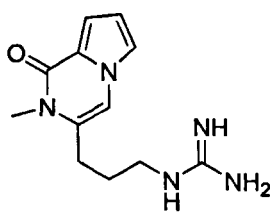
The secondary metabolites isolated from fungal endophytes have different biosynthetic pathways: isoprenoid, polyketide and amino acid derivatives. They belong to different structural categories: alkaloids, peptides, steroids, terpenoids, quinones, flavonoids, aliphatic compounds, and phenols, *etc.*

1.7.1 Alkaloids

Alkaloids are quite common secondary metabolites from endophytes, and some of them show antimicrobial activity.

Amines and amides are quite common isolates from *Acremonium* endophytes which was reclassified as genus *Neotyphodium* [91].

Peramine (**1**), a pyrrolopyrazine alkaloid, was extracted from *Neotyphodium coenophialum*, *N. lolli*, *Epichloë festucae* and from *E. typhina* [92].

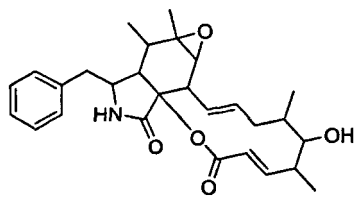


1

This secondary metabolite showed toxicity to insects with no harmful effect on mammals [93-94].

Another group of compounds that possess antitumor and antibiotic activities are the cytochalasins. Cytochalasins are alkaloids which are commonly produced by endophytic fungi such as *Xylaria*, *Phoma*, *Hypoxylon*, and *Chalara* genera [95].

Furthermore, three new cytotoxic cytochalasins (**2-3**), together with the known metabolite cytochalasin E (**4**) were characterized from the culture of *Rhinocladiella* sp., an endophyte present in the perennial twining vine of *Tripterygium wilfordii* [95].



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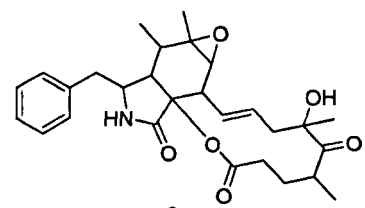

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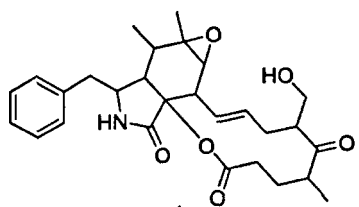

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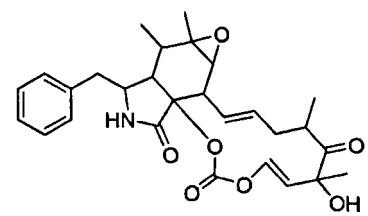
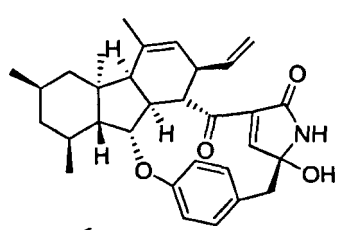


5

Two newly reported antibiotics, pyrrocidines A (**6**) and B (**7**) were isolated from the endophyte *Acremonium zeae* in *maize* and displayed significant antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* [96].



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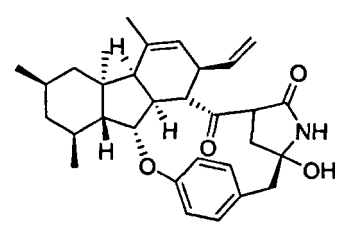
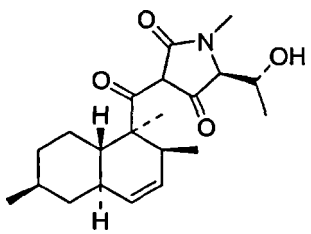


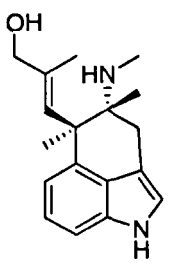
7

Recently, a tetramic acid analogy cryptocin (**8**), an effective antimycotic against *Pyricularia oryzae* and other phytopathogens, was characterized from the culture of an endophytic fungus *Cryptosporiopsis* cf. *Quercina* [97], which was found in the inner bark of the stems of *Tripterygium wilfordii* [98].



8

Indole alkaloids such as chanoclavine (**9**), agroclavine (**10**) and elymoclavine (**11**) were isolated from a culture of *Neotyphodium* endophytes [99].



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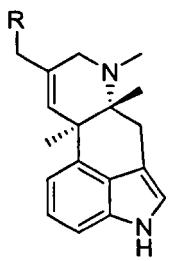
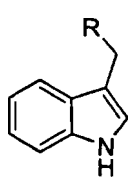


10 agroclavine R= H
11 elymoclavine R= OH

Some endophytes can produce plant hormones with an indole framework [69]. The growth-enhancing phytohormone indole-3-acetic acid (IAA, **12**) was isolated from cultures of an endophytic fungal endophyte *Acremonium coenophialum* [100], *Aureobasidium pullulans* and



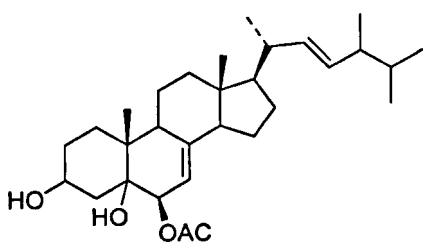
12 R= COOH

Epicoccum purpurascens [101] and *Colletotrichum* sp., an endophytic fungus in *Artemisia annua* [102].

The alternative production of endophytes for phytohormones is assumed to be related to the plant growth-promoting effect allowed by the endophyte infection[69].

1.7.2 Steroids

Two new steroids, 3β-5α-dihydroxy-6β-acetoxyergosta-7,22-diene (**13**) and 3β,5α-dihydroxy-6β-phenyl- *Nat*acetoxyergosta-7,22-diene (**14**) were characterized from the culture medium of an endophytic fungus *Colletotrichum* sp. of *A. annua.* Metabolites 60 and 61 were shown to be antifungal against some crop pathogens *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum* and *Phytophthora capisici* [102].



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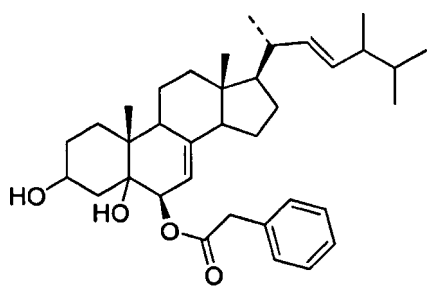


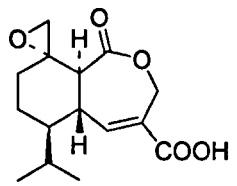
14

Most steroid compounds isolated from endophytes only showed moderate antimicrobial activities, so it appear to be quite difficult to find effective steroid drugs or pesticides from endophyte [103].

1.7.3 Terpenoids

Terpenoids have often been isolated from endophyte cultures which colonize a variety of host plants. Heptelidic acid (**15**) and hydroheptelidic acid (**16**) characterized from *Phyllosticta* sp., an endophytic fungus of *Abies balsamea*, were

found to be toxic to spruce bud worm (*Choristoneura fumiferana*) larvae [104]. Two new benzofuran carrying normonoterpene derivatives (**17**) and (**18**), have been isolated from a culture of an unidentified endophytic fungus obtained from wintergreen *Gaultheria procumbens* [105].



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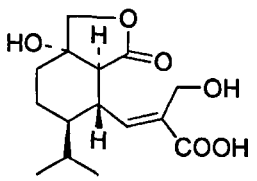

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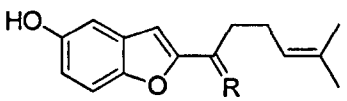
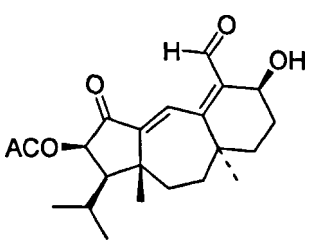


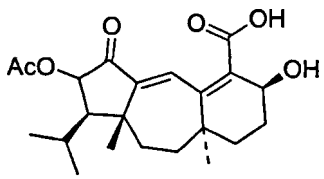
17 R= O
18 R= OH, H

A novel diterpenoid, Guanacastepene (**19**), produced by an unidentified fungus from the branch of *Daphnopsis Americana* growing in Guanacaste, Costa Rica, was found to exhibit antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [106].



19

Subglutinol (**20**), an isolate of *Fusarium subglutinans*, an endophytic fungus from the perennial twining vine *Tripterygium wilfordii* was found to be immunosuppressive but not cytotoxic [107].



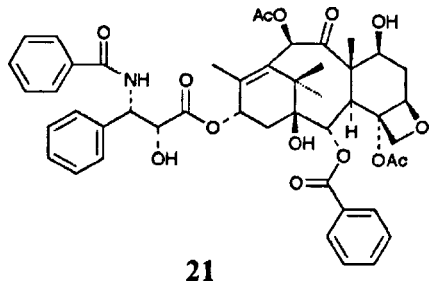
20

Paclitaxel (Taxol (**21**)), a highly functionalized diterpenoid possesses outstanding antioomycete activity, is found in each of the world's yew (*Taxus*) species [89]. Paclitaxel and some of its derivatives are the first major group of anticancer agents that is produced by endophytes [56].

Taxol was firstly isolated in extremely small quantities from the inner bark of the Pacific yew, *Taxus brevifolia,* a slow growing *Taxus* species, is an effective anticancer diterpene [108-111]. *Taxomyces andreanae*, a new endophytic fungus isolated from a Pacific yew *T. brevifolia* in Montana, USA, was the first reported endophyte to produce taxol in 1993.

Electrospray mass spectrum technique was used to prove the existence of this compound in the culture medium of the endophytic fungus, *T. andreanae.* In electrospray mass spectroscopy, paclitaxel usually gives two peaks, one at a mass of 854, which is $(M + H^+)$, and the other at 876, which is $(M + Na^+)$[90]. Since then, a variety of endophytic fungi belonging to different categories isolated from *T. Brevifolia* [112], *T. Wallachiana* [113], *T. yunnanensis*, *T. baccata*, *T. Mairei* [114], *Taxodium distichum* [115-116], *Torreya grandifolia* [117], *Colletotrichum gloeosporioides*

[110] and *Wollemia nobilis* [118-119] have been reported to be able to produce Taxol and/or taxane derivatives in some endophyte cultures.

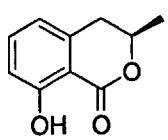


21

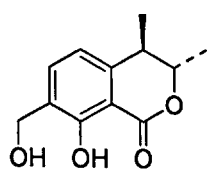
Paclitaxel was also isolated from *Pestalotiopsis microspora* [113, 120], which was the first endophytes residing in plants other than *Taxus* spp , *Pestalotiopsis guepini* [121-122], *Seimatoantlerium tepuiense* [123].

1.7.4 Isocoumarin derivatives

(*R*)-Mellein (**22**), an isocoumarin isolated from *Pezicula* spp. [124], is a powerful fungicidal, herbicidal and algicidal. Gamahorin (**23**) is an isocoumarin characterized from stromata of *E. typhina* on *P. pratense* [125].



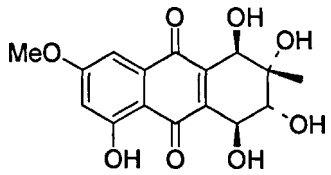
22



23

1.7.5 Quinones

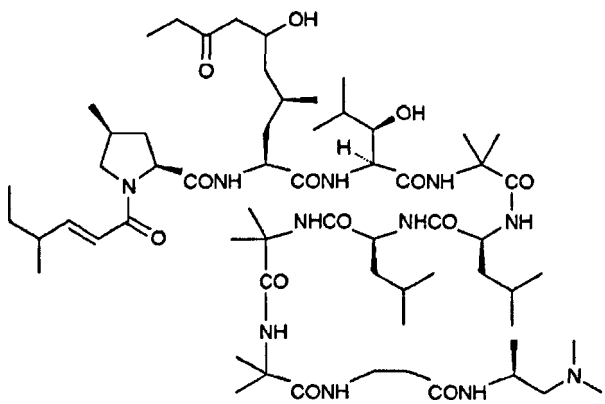
Altersolanol A (**24**), a highly hydroxylated quinone, found in phytopathogenic *Alternaria* cultures, was reisolated from an endophytic *Phoma* sp. and found to be an antibacterial agent [126].



24

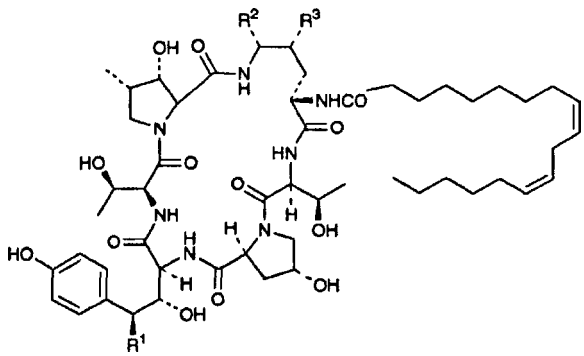
1.7.6 Peptides

Leucinostatin A (**25**), an oligopeptide with phytotoxic, anticancer and antifungal properties characterized originally from *Penicillium lilacinum* [127], was reisolated from culture of *Acremonium* sp., an endophytic fungus from *Taxus baccata* [128].



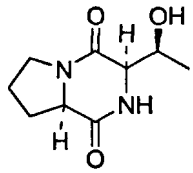
25

The cyclopeptides echinocandins A , B, D and H, were isolated from endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus sylvatica* and shown to be antimicrobial [129]. These metabolites were originally produced by *Aspergillus rugulosus* and *A. nidulans* var. *Echinulatus* [130].



echinocandins A R^1= R^2= R^3= OH
echinocandins B R^1=H, R^2= R^3= OH
echinocandins D R^1= R^2= R^3= H
echinocandins H R^1= R^2= OH, R^3= OMe

Cyclo(Pro-Thr) (**26**) and cyclo(Pro-Tyr) (**27**) were two new antibacterial compounds produced by the fermentation broth of endophytic fungus a *Penicillium* sp. isolated from mangrove plant *Acrostichum aureurm* [131].



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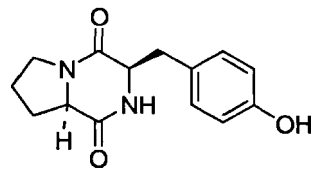
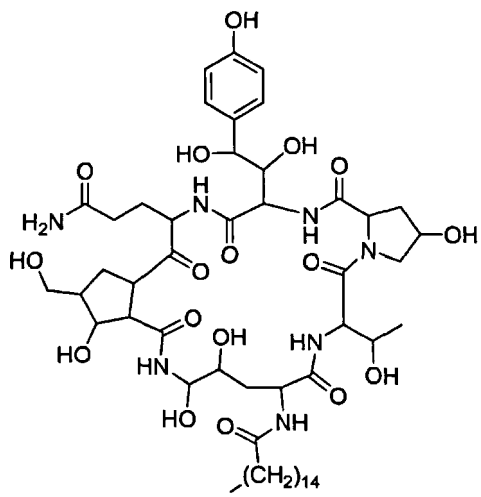


27

A unique peptide antimycotic, termed cryptocandin (**28**), was isolated and characterized from *C. quercina*, a fungus isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia [98].

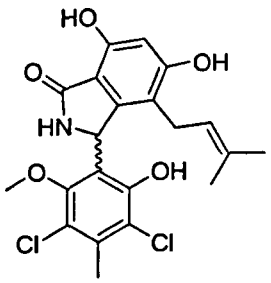


28

1.7.7 Phenols

Phenol and phenolic acids have often been isolated from endophyte cultures originating from a variety of host plants [103].

Pestalachlorides A–C (**29-31**), three new chlorinated benzophenone derivatives, have been isolated from cultures of an isolate of the plant endophytic fungus *Pestalotiopsis adusta.* Pestalachlorides A and B displayed significant antifungal activities against three plant pathogens [132].



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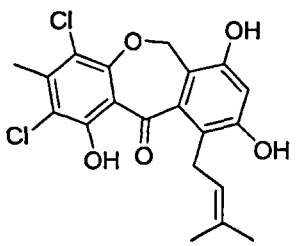

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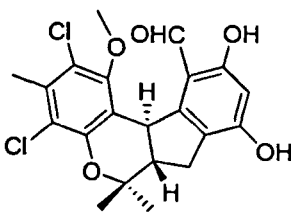
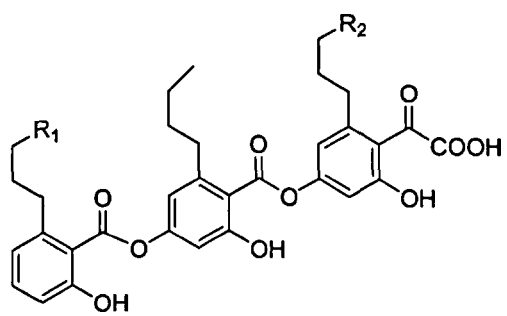


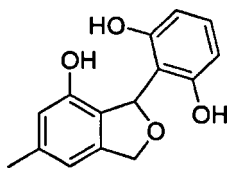
31

Another fascinating use of secondary metabolites from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors, cytonic acids A (**32**) and B (**33**), have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures as *p*-tridepside isomers were elucidated by mass spectrometry and NMR methods [133].

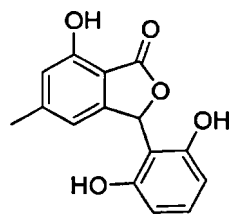


32 Cytonic acid A R1 = Et, R2 = H
33 cytonic acids B R1 = H, R2 = Et

Endophytic fungi can produce compounds with antimicrobial as well as antioxidant activity. Pestacin (**34**) and isopestacin (**35**), have been isolated from culture medium of *P. microspora*, an endophyte isolated from a plant called *Terminalia morobensis* growing in the Sepik River drainage of Papua New Guinea [134]. Both pestacin (**34**) and isopestacin (**35**) display antimicrobial as well as antioxidant activity [134].



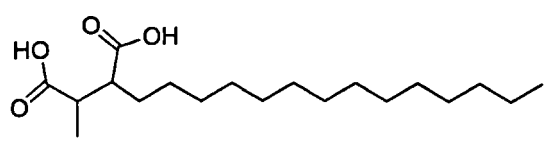
34



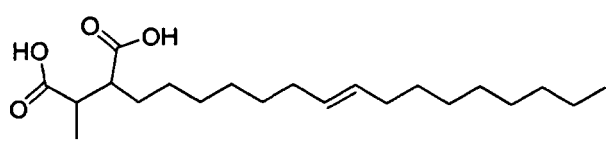
35

1.7.8 Others

Chaetomellic acids A (**36**) and B (**37**) isolated from the culture of an endophytic *Chaetomella acutisea* were found to be specific inhibitors of farnesylprotein transferase (FPTase) [135].

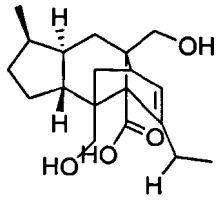


36



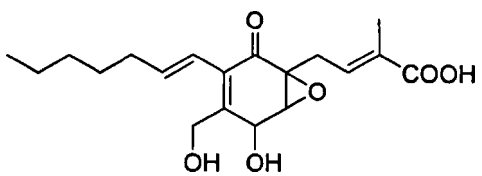
37

Sordaricin (**38**) cultured by the endophytic fungus *Xylaria* sp. isolated from the leaves of *Garcinia dulcis*, exhibited moderate activity against a wide range of fungal pathogens [136].



38

Ambuic acid (**39**), a highly functionalized cyclohexenone isolated from *P. microspora*, possesses antifungal activity[137]. This compound has also been used as a model to develop new solid-state NMR experiments for the structural determination of organic substances [138-139].



39

There is mounting evidence that many bioactive compounds isolated from plants, as well as marine and terrestrial invertebrates, are actually metabolites produced by symbiotic microorganisms [140]

1.8 The aims of the project

This project is designed to achieve the following objectives:

- Create secondary metabolite profiles of the studied endophytic *Xylaria* fungi.
- Search for novel and bioactive secondary metabolites produced by endophytic *Xylaria* fungi
- Apply NMR and other spectroscopic techniques to elucidate the structure of isolated secondary metabolites.

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Chapter 2: Secondary metabolites from X. A311R

2.0 Fungus A311R overview

Endophytic fungus A311R was collected from palm tree leaves *Arenga pinnata* (Sugar Palm) from Kao Loung National Park in Thailand.

Arenga pinnata Sample leaf of *Arenga pinnata*

Figure 2 *Arenga pinnata* palm tree

The fungus was received from Prof A.J.S Whalley, Liverpool John Moores University, as cultures in Petri dishes as shown in Figure 3. The examination of the fungus for its secondary metabolites is discussed in this chapter.

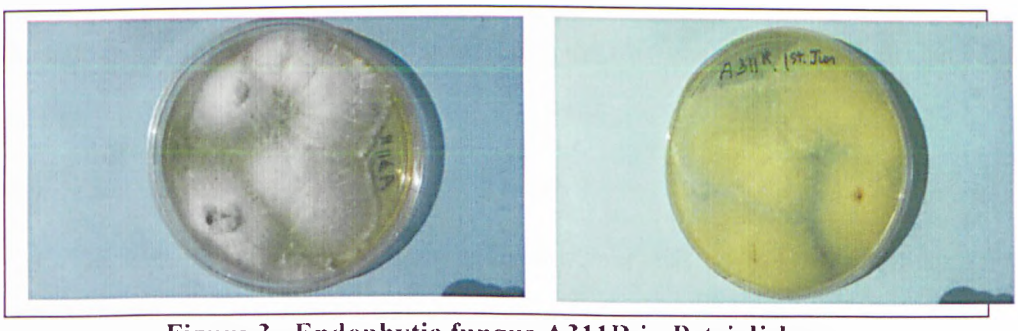


Figure 3 Endophytic fungus A311R in Petri dishes.

The culture of endophyte A311R was sub-cultured in four conical flasks containing 3% aqueous malt extract for 3 weeks. Fruiting bodies developed after 10 days. The light brown fruiting bodies grew 1.0-1.5 cm high with a base diameter of 0.5 mm on the black and white upper surface of the mycelium. The mycelium also developed white ball-shaped bodies as well (Figure 4).

The cultured endophyte was then transferred into twelve Thompson bottles and allowed to grow on 3% aqueous malt extract for 8 weeks. Master cultures of these endophytes were made for future reference. The initially white mycelium (4 weeks) later turned brittle and black on the underside and supported un-branched, light brown-tipped xylaria-type fruiting bodies.

Figure 4 A311R endophytes after ten days

2.1 Isolation and structure elucidation of nonane-1,2,3-tricarboxylic acid (2.1)

The mature cultures (12 L) were harvested and the mixture filtered through a muslin cloth to recover the mycelium. The aqueous filtrate was extracted in batches with ethyl acetate in a separating funnel (5 L) and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate. Removal of the solvent by rotary evaporation gave brown gummy material (4.32 g). The latter was dissolved in warm

chloroform and set aside for 24 h. A light brownish solid (0.90 g) precipitated from the solution. The solid was recovered by filtration and crystallised from nitromethane to give nonane-1,2,3-tricarboxylic acid (**2.1**) as yellowish white crystals (375 mg), mp (117-120 °C), ESI $[M-H]^-$ *m/z* 259.1177, $[\alpha]_D^{20}$ – 186° (*c* 1.0, MeOH) $IR_{(ATR)}$ ν_{max} cm^{-1} : 3091, 1713, 1659 and 1175. HRESIMS analysis of **2.1** gave a molecular ion at *m/z* 259.1177 in the HR-ESIMS spectrum is compatible with the formula $C_{12}H_{20}O_6$. The prominence of the M-18 peak (*m/z* 241.1) in the ESIMS spectrum of **2.1** is indicative of the presence of a hydroxyl group [1], which is supported by the IR absorption at 3091 cm^{-1} (Figure 5). The molecular formula suggested three degrees of unsaturation, which are possibly due to carbonyl groups, which show absorptions at 1713, and 1659 cm^{-1}.

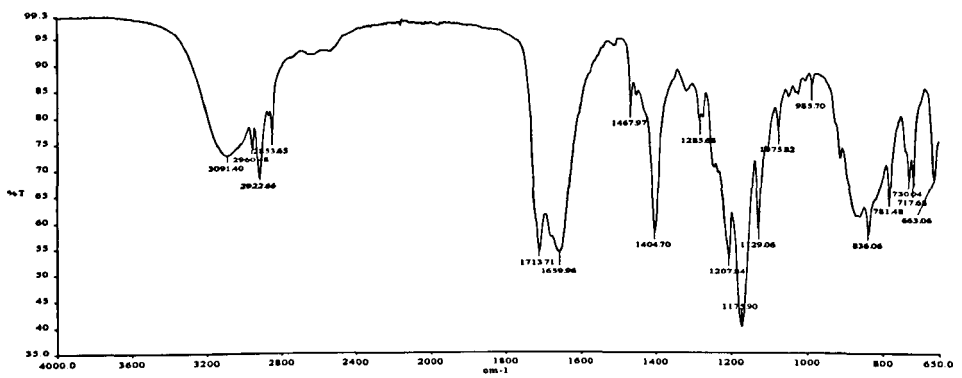


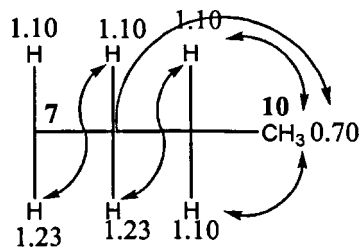
Figure 5 IR spectrum of 2.1

^1H NMR spectrum (C_5D_5N) (Figure 11) showed a methyl triplet at δ_H 0.70 (3H, t, *J* 6.5 Hz), methylene protons at δ_H 1.10 (4H, m), 1.23 (2H, m), 1.47 (1H, m), 1.62 (1H, m), 1.86 (1H, m), 2.14 (1H, m), 3.46 (1H, m) , 3.23 (1H, dd *J* 3.6, 14.0) and two methine protons at δ_H 3.50 (1H, m) and 4.12 (1H, m).

The ^{13}C (Figure 12) and DEPT 135 NMR spectra (Figure 13) run in deuterated pyridine revealed 12 carbons: one methyl at δ_C 14.04, six methylene signals at δ_C 22.69, 28.32, 29.12, 29.49, 31.77 and 33.94, two methine carbon signals at δ_C 44.34 and 47.42 and three quaternary carbons at δ_C 175.50, 176.53 and 177.00. The last 3 quaternary carbons could be carboxylic acid or ester carbonyls. The IR absorptions at 3091 and 1713 cm^{-1} supported carboxylic acid carbonyl groups.

The HMQC NMR (Figure 14) and DEPT-135 spectra (C_5D_5N) allowed the assignment of all the protons and their coupling carbon signals (Table 3)

Analysis of the correlations in the ^1H-^1H COSY (Figure 15) and HMBC NMR spectra (Figure 16) allowed the establishment of partial substructure **2.1a**. In the ^1H-^1H COSY spectra, H-7b at δ_H 1.23 was coupled to H-8a at δ_H 1.10 and in addition H-9b at δ_H 1.10 was coupled to H-10 at δ_H 0. 70. The HMBC was useful in the assignment of C-7, C-8 and C-9 because of the two and three bond correlations between the protons of the methyl group at δ_H 0.70 and the methylene carbon C-8 at δ_C 31.77 (Figure 6).



Green arrows: ^1H-^1H COSY
Red arrows: HMBC

Figure 6 Substructure 2.1a

In the ^1H-^1H COSY spectrum, H-7a at δ_H 1.10 is coupled to H-6a at δ_H 1.47 and H-6b at δ_H 1.62, which is further coupled to H-5a at δ_H 2.14 and H-5b at δ_H 1.86

respectively. Furthermore H-5a is coupled to the H-4 methine multiplet at δ_H 3.50 which also has coupling with the H-3 methine multiplet at δ_H 4.12. The H-2a resonating at δ_H 3.46 is coupled to H-3 methine, thus completing the network of coupling throughout the molecule (Figure 7).

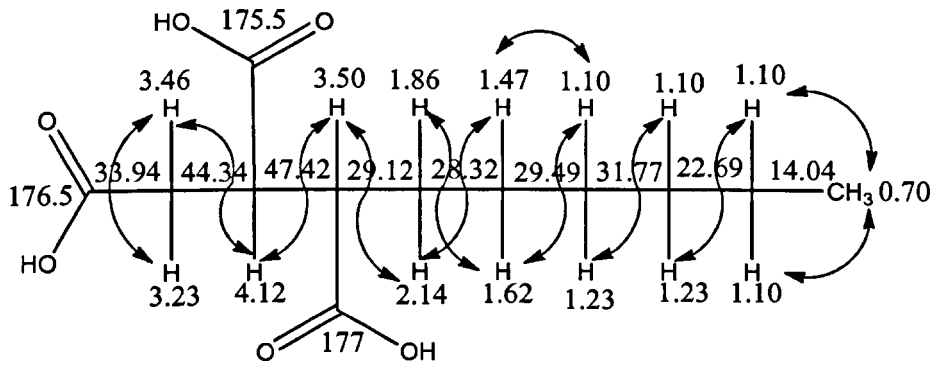
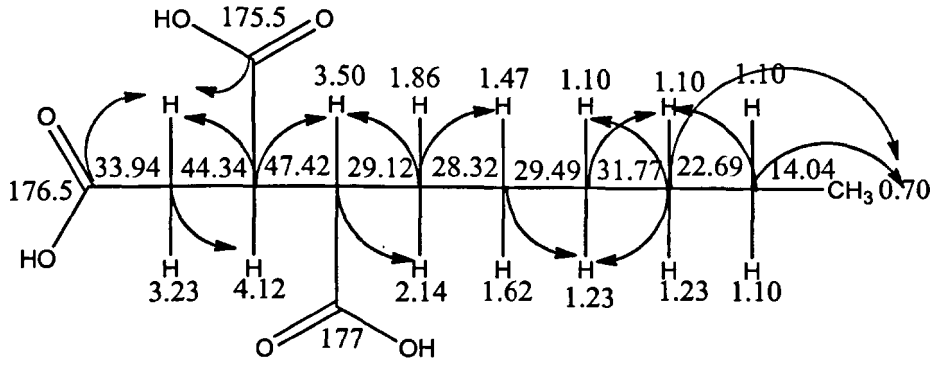


Figure 7 ^{1}H-^{1}H correlation network throughout compound 2.1

The HMBC spectrum of **2.1** also showed cross peaks between δ_H 3.46 (H-2a) and the signals at δ_H 175.5 (C-11) and 176.5 (C-1) confirming their positional assignments.

The H2BC spectrum (Figure 17) was also used to track the two bond C-H correlations throughout the structure. H2BC together with HMQC experiments provides a map of one and two bond correlations [2]. The connectivity was easily followed throughout the structure. C-2 correlates to H-3, C-3 correlates to H-4, C-4 correlates to H-5 and C-5 correlates to H-6, *etc* (Figure 8).



Blue arrows: H2BC Red arrows: HMBC

Figure 8 H2BC connectivity of compound 2.1

Table 3 Full assignment of the protons and their coupling to carbon signals in compound 2.1

No	δ_C	δ_H	H2BC	H-H COSY	HMBC
1	176.53 – C				3.23, 3.46
2	33.94 - CH_2	2a: 3.46 (m) 2b: 3.23 (dd *J* 3.6, 14.0)	4.12	3.23, 4.12	
3	44.34 – CH	4.12 (m)	3.46, 3.50	3.5, 3.46	
4	47.42 – CH	3.50 (m)	2.14	4.12, 2.14,	
5	29.12 - CH_2	5a: 1.86 (m) 5b: 2.14 (m)	3.50, 1.10, 1.47	1.47, 1.62, 1.86	
6	28.32 - CH_2	6a: 1.47 (m) 6b: 1.62 (m)	1.23	1.47	
7	31.77 - CH_2	7a: 1.10 (m) 7b: 1.23 (m)	1.23, 1.10		0.70, 1.10, 1.23
8	29.49 - CH_2	8a: 1.10 (m) 8b: 1.23 (m)			
9	22.69 - CH_2	9a: 1.10 (m) 9b: 1.10 (m)	1.10, 0.70	0.70	0.70, 1.10
10	14.04 - CH_3	0.70 (3H, t, *J* 6.5)	1.10	1.10	
11	175.5 – C				
12	177.00 – C				

This spectral data identifies compound **2.1** as nonane-1,2,3-tricarboxylic acid (Figure 9).

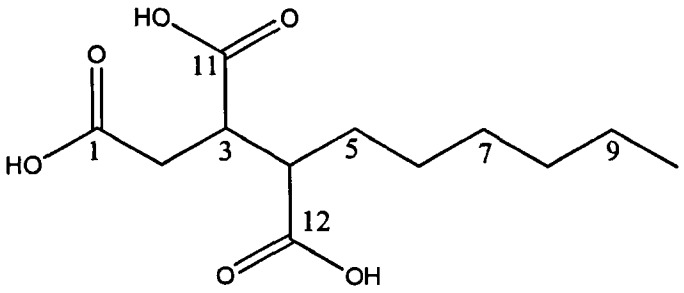


Figure 9 Nonane-1,2,3-tricarboxylic acid (2.1)

This is the first isolation of this compound from a natural product source. It is also the first report of the NMR data for nonane-1,2,3-tricarboxylic acid. This compound was synthesized for the first time in 1950 (mp 150 °C) for bioactivity studies, but there was no NMR data reported [3]. It was found to be inactive against *M. Tuberculosis* bacteria [3].

Jayasuriya *et al* in 1996 reported the isolation of a related compound, oreganic acid ($C_{22}H_{38}O_{10}S$) from an endophytic fungus isolated from living leaves of *Berberis oregano,* which has the structure shown in Figure 10 [4]. It was found to exhibit anticancer properties [4].

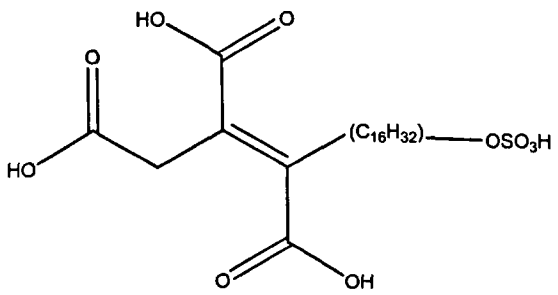


Figure 10 Structure of Oreganic acid

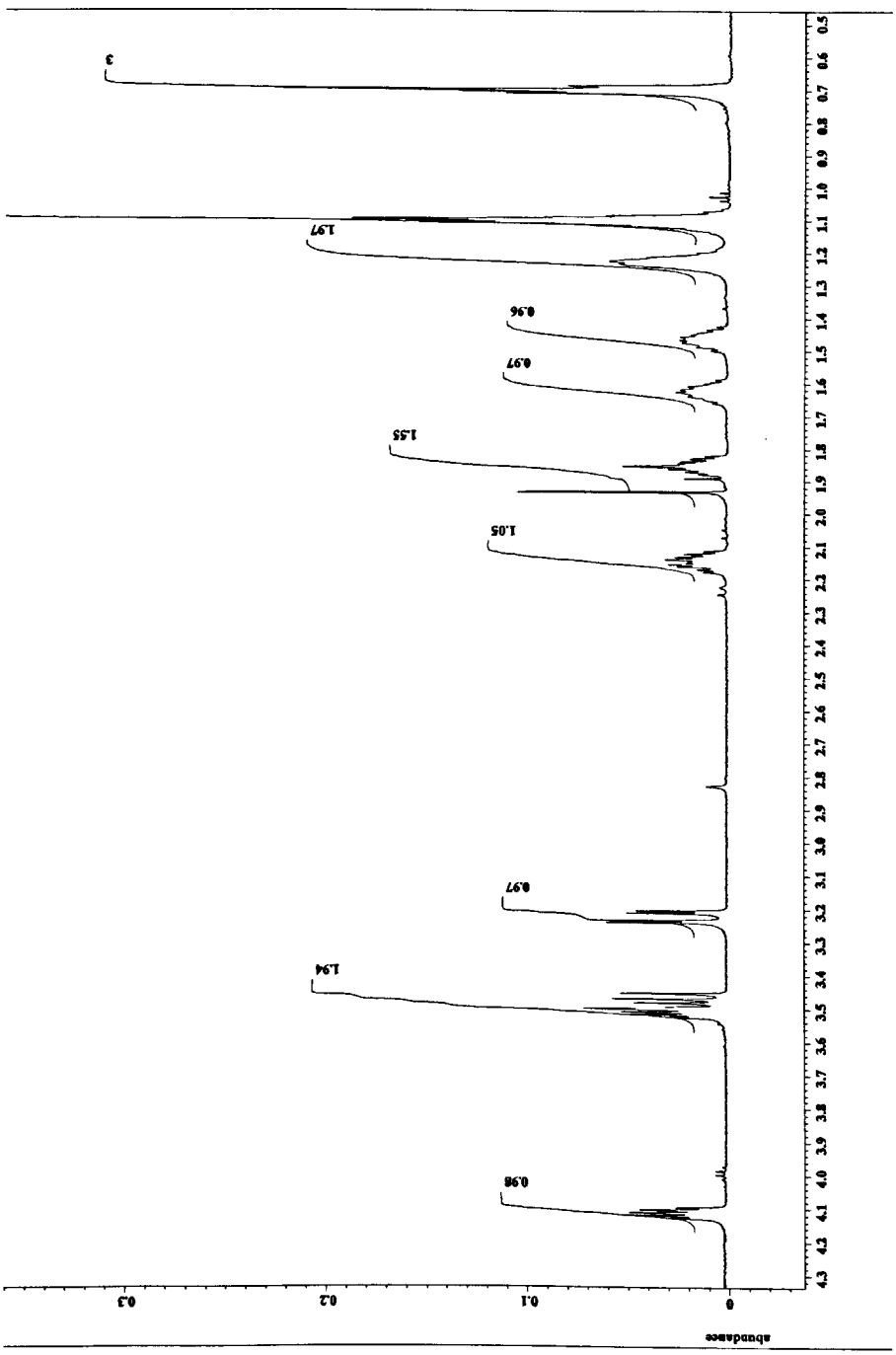


Figure 11 ^{1}H NMR spectrum (C_5D_5N) of **2.1**

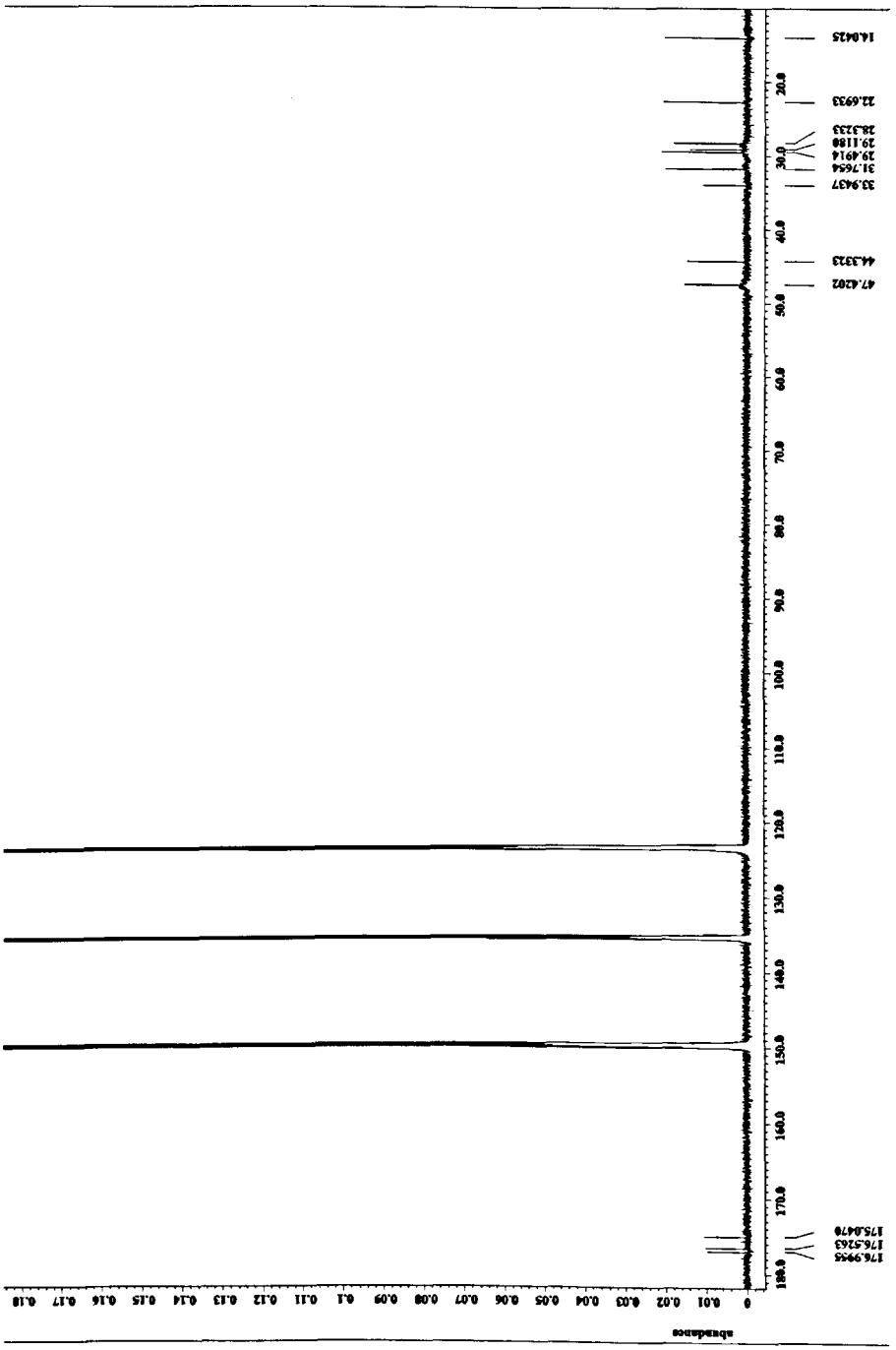


Figure 12 ^{13}C NMR spectrum (C_5D_5N) of 2.1

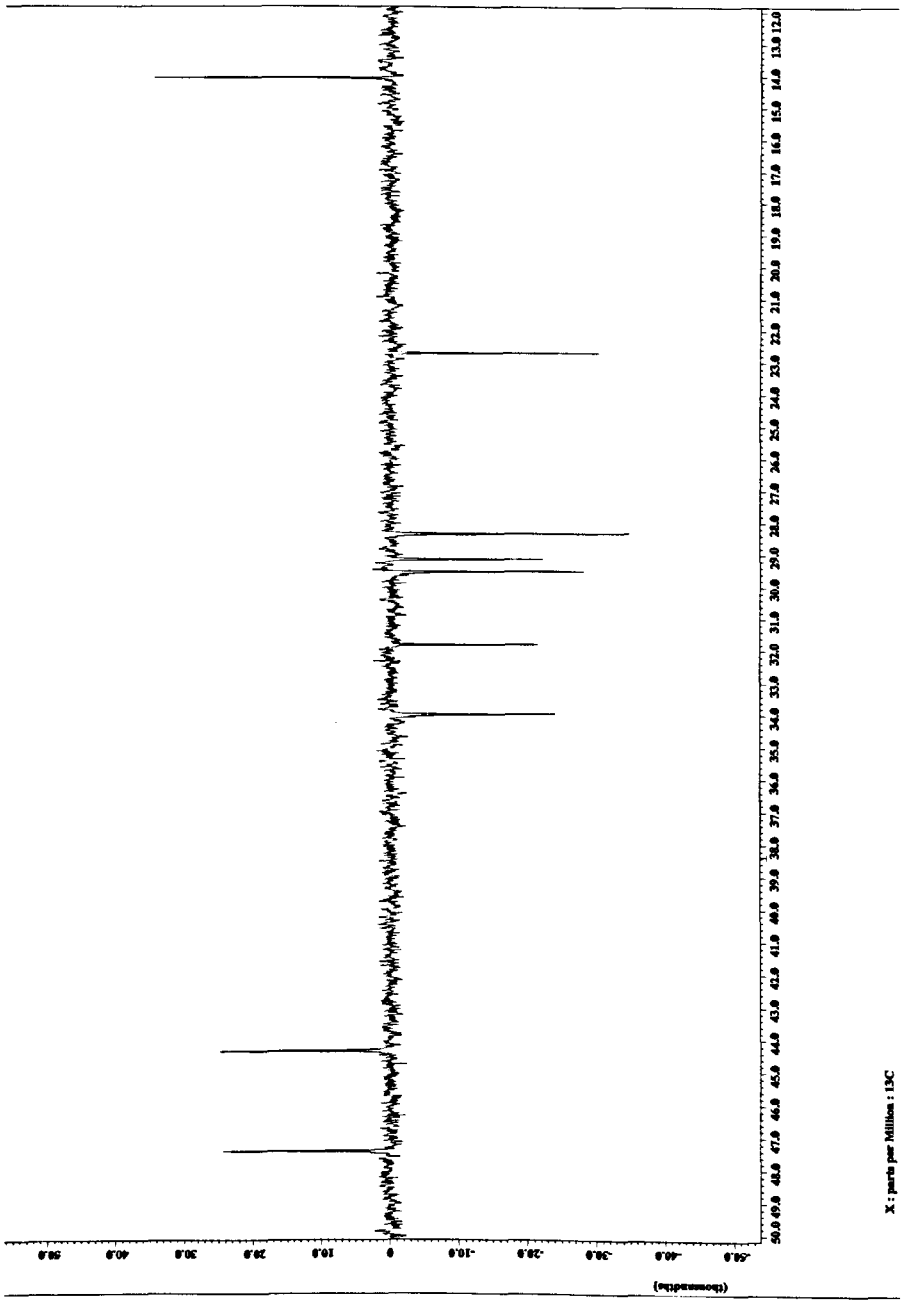


Figure 13 DEPT-135 NMR spectra of 2.1

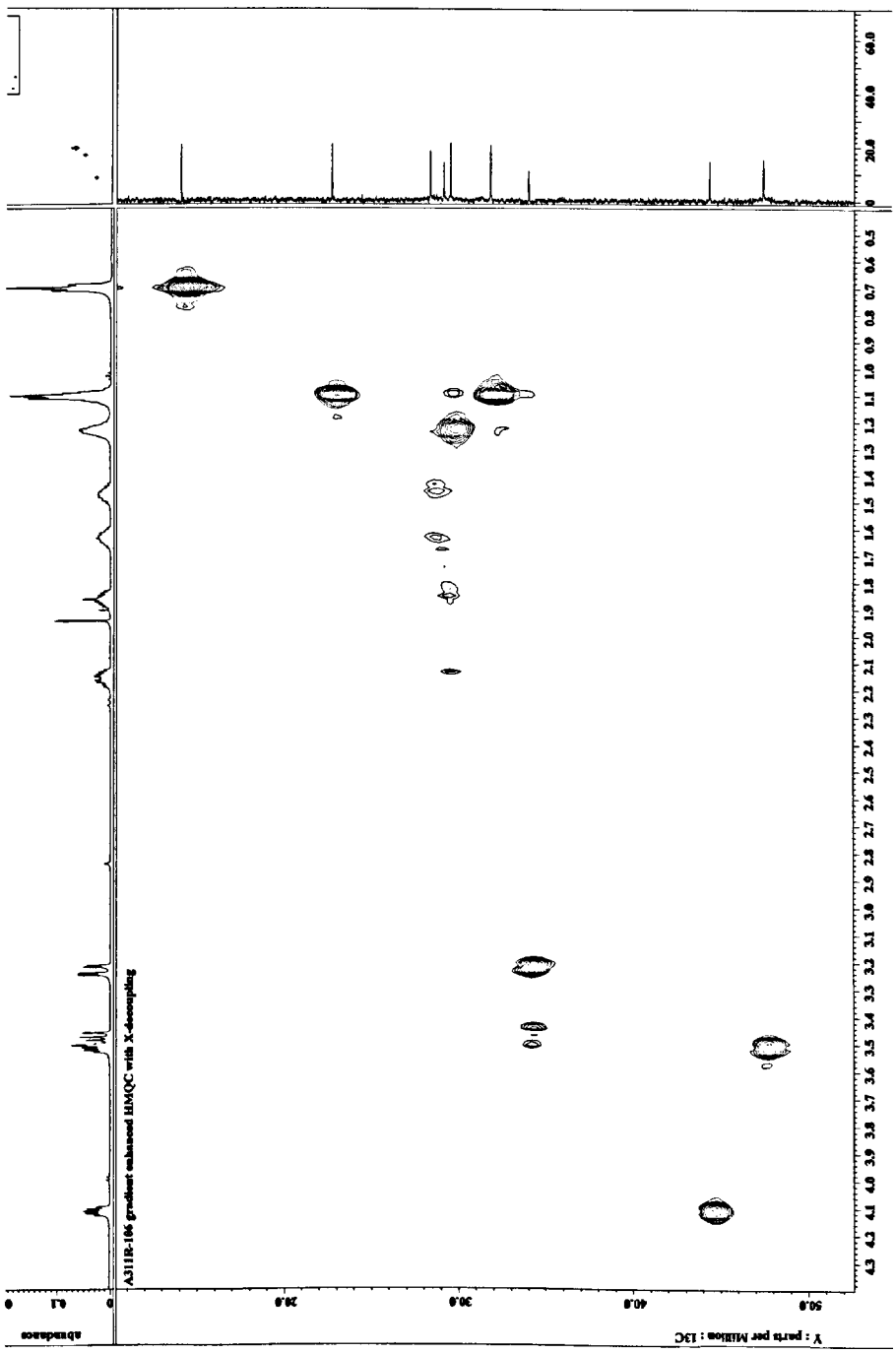


Figure 14 HMQC NMR spectrum of 2.1

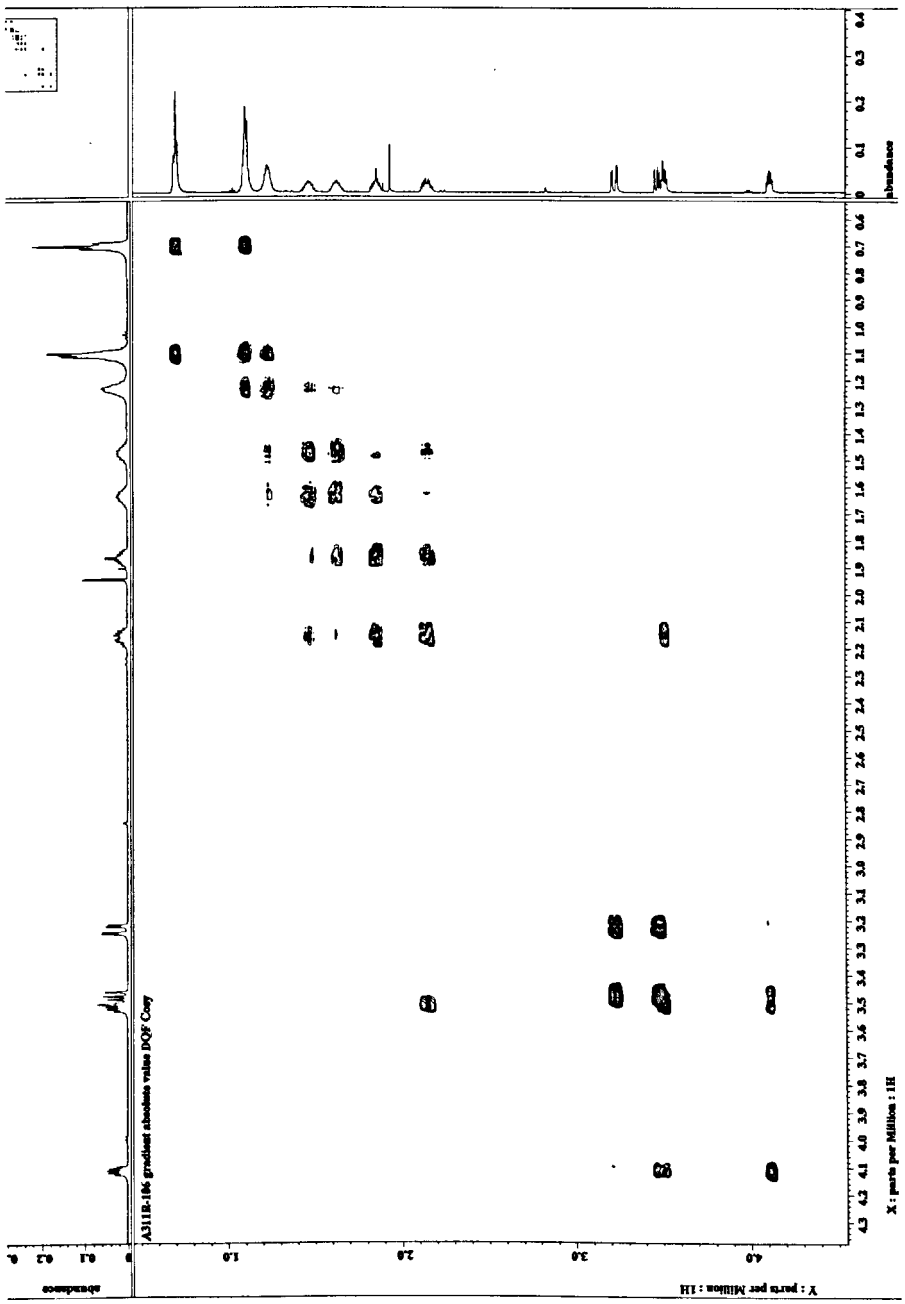


Figure 15 ^{1}H-^{1}H COSY NMR spectrum of 2.1

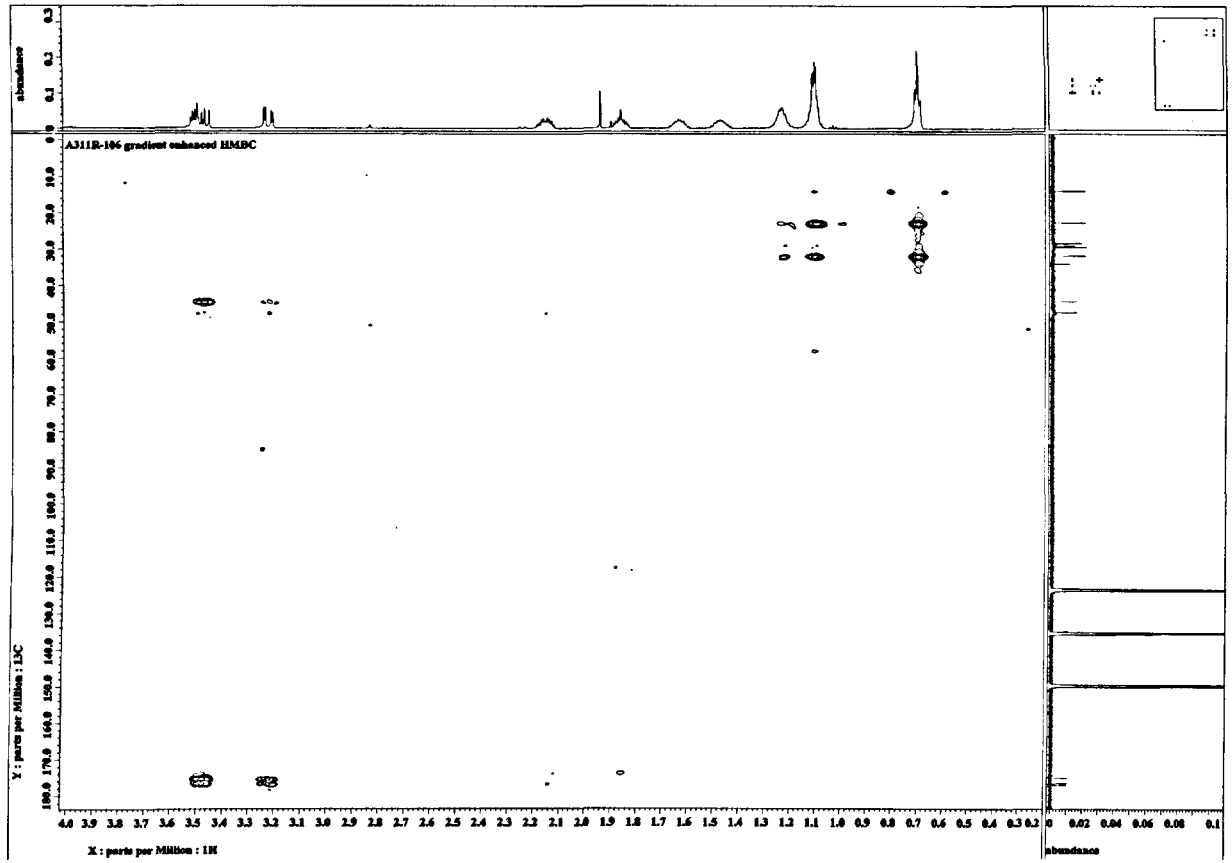


Figure 16 HMBC NMR spectrum of 2.1

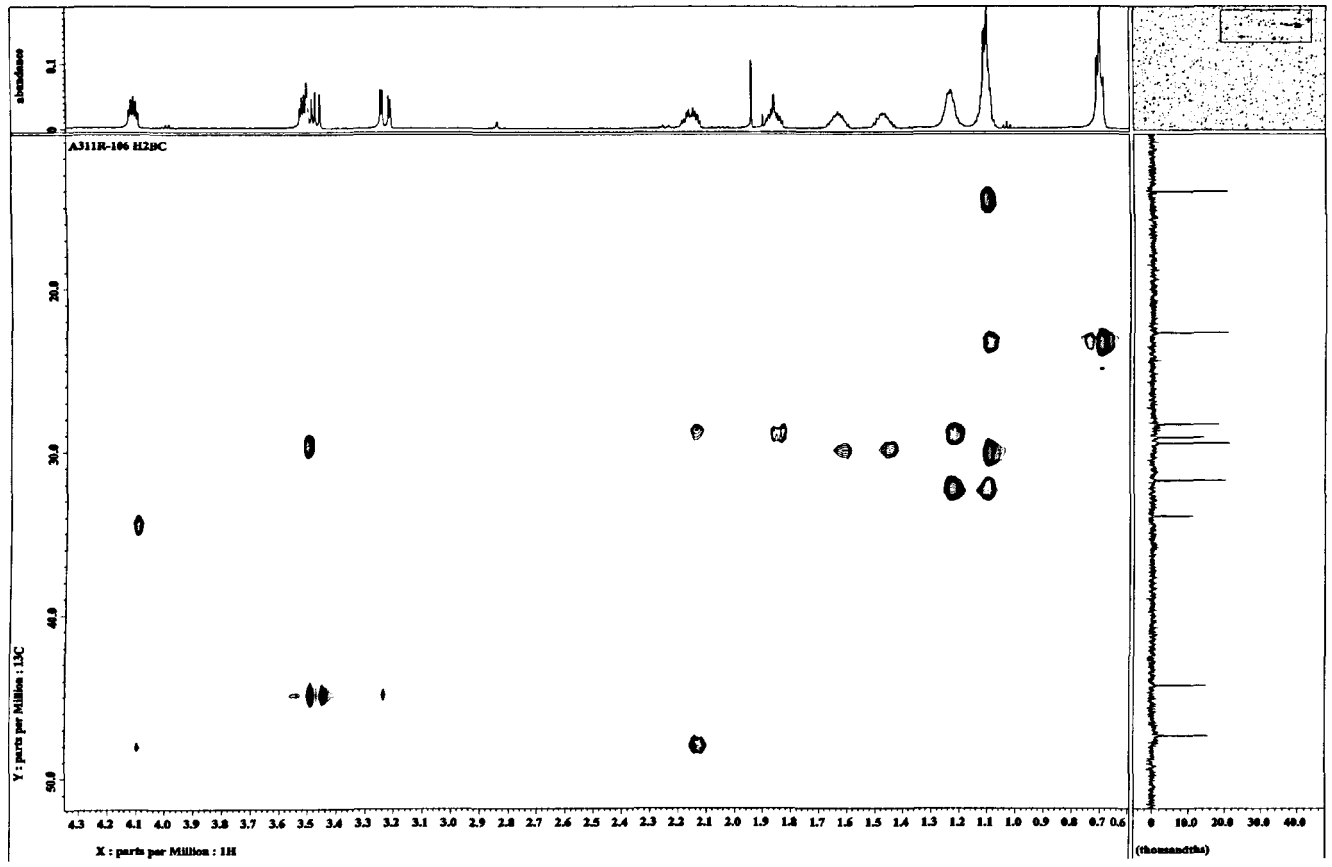
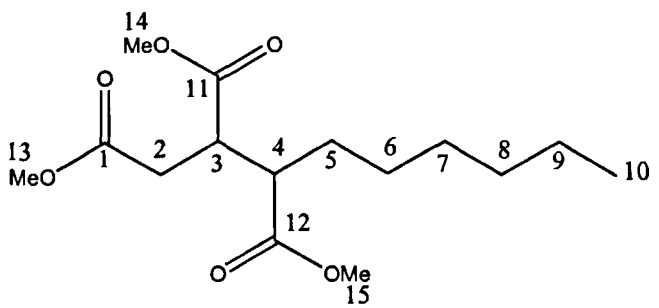


Figure 17 H2BC NMR spectrum of 2.1

2.1.1 Trimethyl nonane-1,2,3-tricarboxylate (2.1A)

To confirm the structure of **2.1**, the methyl ester derivative was prepared. Reaction of **2.1** with diazomethane gave a trimethyl ester (**2.1A**) confirming the presence of 3 carboxylic acid groups in the parent molecule. The ester (**2.1A**) gave a molecular ion at ES $[M+H]^+$ *m/z* 303 which is consistent with the molecular formula $C_{15}H_{26}O_6$. The mass spectrum also showed a strong ion at *m/z* 271 corresponding to the loss of a methoxy group from the parent ion. The IR spectrum showed the absence of carboxylic groups and the appearance of an ester stretching absorption at 1732 cm^{-1}. The ^1H NMR spectrum (C_5D_5N, Figure 18) showed the protons at δ_H 0.72 (3H, d, *J* 7.0 Hz), 1.08 (6H, m), 1.23 (2H, m), 1.49 (1H, m), 1.73 (1H, m), 2.77 (1H, dd, *J* 4.3, 12.5), 2.92 (2H, m), 3.43 (1H, m), 3.52 (3H, s), 3.59 (3H), 3.61 (3H, s).

The ^{13}C NMR spectrum of **2.1A** (Figure 19) showed three carbonyl signals at δ_C 172.28, 173.50 and 174.25, which represent shifts of approx. 3 ppm to higher field compared to their positions in the parent molecule.



2.1A

Compared with the parent, the ^1H NMR spectrum shows 3 extra signals corresponding to the methoxy protons at δ_H 3.52, 3.59 and 3.61, which in turn

showed cross-peaks with δ_C 51.58, 51.65 and 51.92 respectively in the HMQC NMR spectrum.

The HMBC correlations between the methyl ester protons and their adjacent carbonyls allowed the assignments of their corresponding carbonyls. Similarly the HMBC correlations between the carbonyl groups and the 2 and 3 bond neighbouring protons helped to confirm their placement within the molecule. The ^1H and ^{13}C NMR spectral data of trimethyl nonane-1,2,3-tricarboxylate **(2.1A)** are summarized in Table **4**.

Table 4 The ^1H and ^{13}C NMR spectral data of trimethyl nonane-1,2,3-tricarboxylate2.1A

No	δ_C	δ_H	HMBC
1	174.25 – C		3.61
2	33.26 - CH_2	2a: 2.91 2b: 2.77 (dd, *J* 4.3, 12.5)	
3	43.24 – CH	3.43 (m)	
4	46.52 – CH	2.91(m)	
5	29.10 - CH_2	5a: 1.49 (m) 5b: 1.73 (m)	
6	27.46 - CH_2	6a: 1.23 (m) 6b: 1.08 (m)	
7	29.13 - CH_2	7a: 1.08 (m) 7b: 1.08 (m)	
8	31.56 - CH_2	8a: 1.08 (m) 8b: 1.23 (m)	
9	22.59 - CH_2	9a: 1.08 (m) 9b: 1.08 (m)	
10	13.98 - CH_3	0.72 (3H, t, *J* 7.0)	
11	172.28 – C		3.52, 2.91, 2.77
12	173.50 – C		3.59, 1.49, 1.73, 3.43
13	51.92 - OCH3	3.61 (s)	
14	51.58 - OCH3	3.52 (s)	
15	51.65 - OCH3	3.59 (s)	

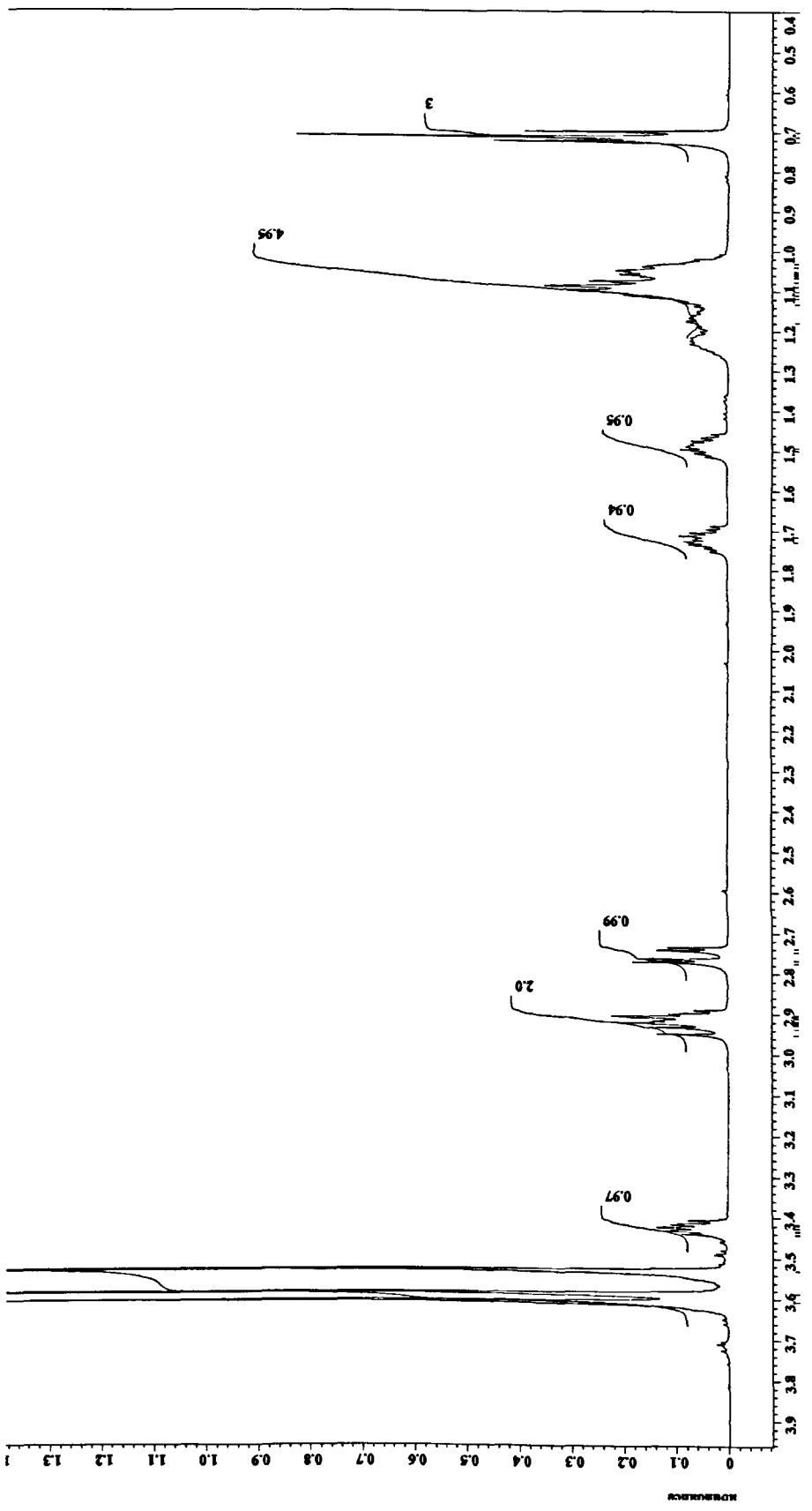


Figure 18 ^{1}H NMR spectrum (C_5D_5N) of 2.1A

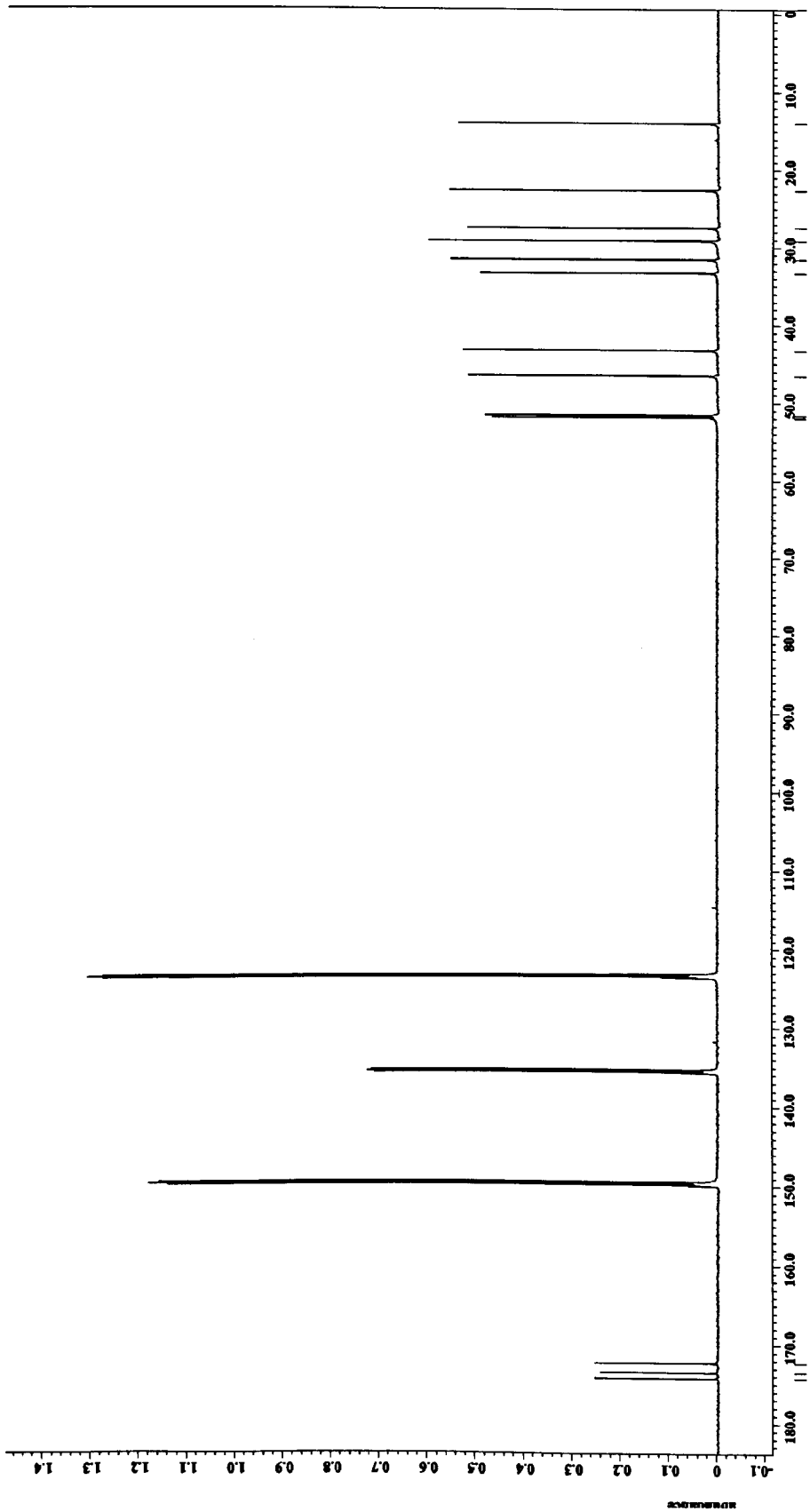


Figure 19 ^{13}C NMR spectrum (C_5D_5N) of 2.1A

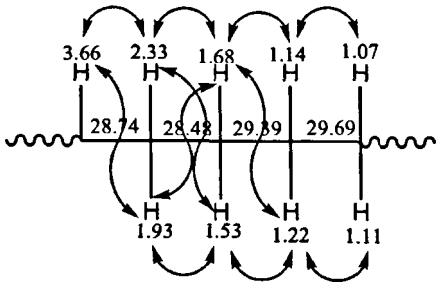
2.2 Isolation and characterisation of spiculisporic acid (2.2)

The crude extract of A311R in addition to the previously described compound, also showed a brown spot near the base of the silica gel plate in the solvent system toluene : ethyl acetate : acetic acid (50:49:1), when sprayed with *p*-nitroaniline spray reagent. 1 g of the ethyl acetate extract was applied to preparative silica plates (5 × 20 cm × 20 cm) to isolate this compound. Ethyl acetate : acetic acid (99:1) was used as the mobile phase to develop the plate. The brown spot moved further up to R_f 0.3. The band was then removed and washed with ethyl acetate to give a light brown solid, which was crystallised from nitromethane to give compound **2.2** as colourless crystals (28 mg), mp 142-145 °C, ESI $[M-H]^-$ *m/z* 327.1809, $[\alpha]_D^{20}$ - 12° (c 0.25, EtOH)

This compound gave a strong negative molecular ion at *m/z* 327 in the ESI mass spectrum, which did not appear to be consistent with the NMR data. The NMR data of 2.2 showed some similarities and differences with that of nonane-1,2,3-tricarboxylic acid (**2.1**). Initial analysis of the ^{13}C NMR spectrum (C_5D_5N) (Figure 23) suggested 12 signals, however the expansion of the area between δ_C 28-31 revealed four additional signals indicative of a molecule containing 16 carbon atoms, which was still inconsistent with mass spectrum negative molecular ion at 327.2. Further processing of the ^{13}C NMR spectrum confirmed that the signal at δ_C 29.69 was in fact two signals overlapping each other. So the ^{13}C, ^1H, HMQC and DEPT135 NMR spectra of **2.2** revealed a molecular formula $C_{17}H_{28}O_6$, which requires a molecular ion at *m/z* 328 which matches the strong molecular ion at m/z 327 seen in the ESI negative mass spectrum.

The ^{13}C NMR spectrum (Figure 23, C_5D_5N) showed 3 carbonyl signals at δ_C 174.52, 175.12 and 176.82 similar to those found in **2.1,** which suggested that this compound could be another tricarboxylic acid metabolite. The main differences in the ^{13}C NMR spectra of **2.1** and **2.2** is the presence in **2.2** of signals at δ_C 52.14 and 87.62 instead of the signals at δ_C 44.34 (C-3) and 47.42 (C-4) in the **2.1.** These new down field signals indicate a strong deshielding effect resulting from the presence of an electronegative atom or a group such as a hydroxyl group. The presence of such a group needs a molecular ion of *m/z* 346, which is not seen in the mass spectrum. In the IR spectrum compound **2.2** showed absorptions typical of carboxylic acid hydroxyl groups and carbonyl groups at 2952, 2550, 1791, 1778 and 1710 cm^{-1}. These groups were also observed as three carbonyl signals at δ_C 174.52, 175.12 and 176.82 in the ^{13}C NMR spectrum. The absorption at 1791 cm^{-1} in the IR spectrum suggested the presence of a five ring lactone system [5] and led to a molecular formula of $C_{17}H_{28}O_6$, which is in turn consistent with the negative molecular ion at *m/z* 327.

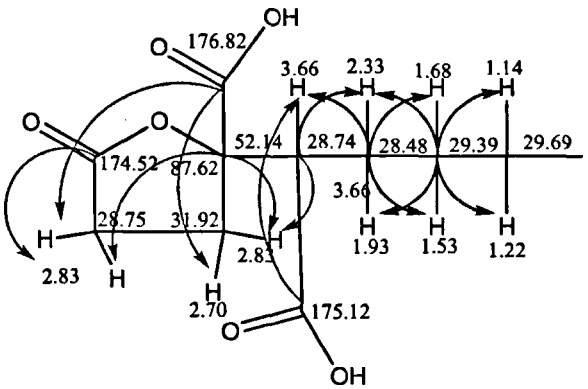
The ^1H NMR spectrum of **2.2** (Figure 22, **C_5D_5N**) showed a prominent doublet of a doublet signal at δ_H 3.66 which showed a cross-peak with δ_C 52.14 in the HMQC spectrum (Figure 25). In the ^1H-^1H COSY spectrum (Figure 26), this signal is coupled to δ_H 2.33 and 1.93, which were further coupled to signals at δ_H 1.68 and 1.53. The last two protons showed further coupling to signals at δ_H 1.14 and 1.22. This analysis of the coupling network in the ^1H-^1H COSY and HMQC NMR spectra allowed the establishment of the partial substructure **2.2a.**



Green arrows: ^{1}H-^{1}H COSY

2.2a

Analysis of the HMBC and H2BC spectra (Figure 28) yielded further connectivities to the substructure **2.2a** to give **2.2b**.



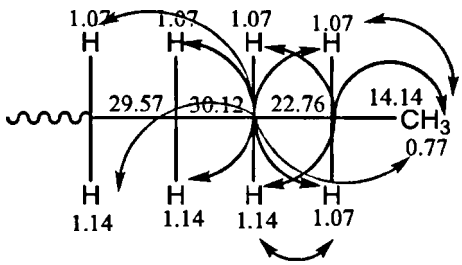
Pink arrows: HMBC
Blue arrows: H2BC

2.2b

In the HMBC spectrum (Figure 27), the signal at δ_H 3.66 showed a cross-peak with δ_C 175.12, the signals at δ_H 2.70 and 2.83 showed cross-peaks with the signals at δ_C 52.14, 176.82 through 3 bonds and the signal at δ_C 174.52 confirming the position of each carbonyl within the molecule.

The ^{1}H NMR spectrum indicated one methyl group at δ_H 0.77 which showed a cross-peak with the signal at δ_C 14.14 in the HMQC. The ^{1}H-^{1}H COSY spectrum showed this signal coupled to δ_H 1.14 and 1.07. The last proton signal at δ_H 1.07 in the ^{1}H NMR spectrum showed an integration of approximately 9 protons. This made the

assignment of connected carbons in the HMQC quite difficult especially between δ_C 28.48-29.69 where there are 8 carbon signals. The HMBC correlation between the methyl protons and the signal at δ_C 30.12 was helpful in the assignment of the substructure **2.2c**.



Green arrows: ^{1}H-^{1}H COSY
Pink arrows: HMBC
Blue arrows: H2BC

2.2c

Full analysis of ^{1}H, ^{13}C, HMQC, HMBC and H2BC spectrum (Table 5) led to the establishment of the final structure of **2.2** to be as shown by Figure 20.

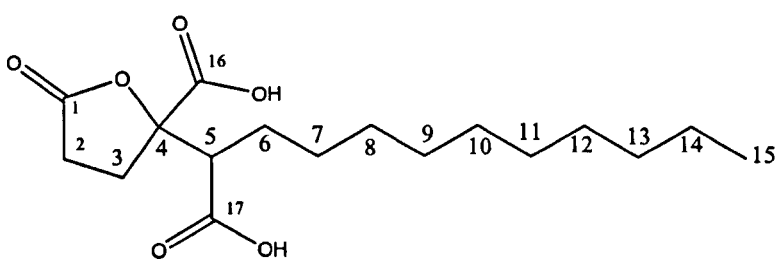


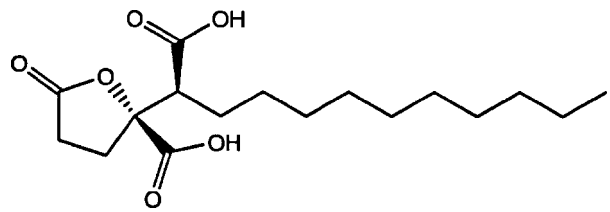
Figure 20 Compound 2.2 structure

Table 5 Full analysis of ^{1}H, ^{13}C, HMQC, HMBC and H2BC NMR data of 2.2 run in pyridine (C_5D_5N)

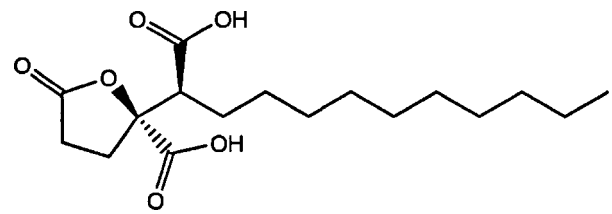
	δ_C	δ_H	H2BC	H-H COSY	HMBC
1	176.82 – C				2.70, 2.83
2	28.75 - CH_2	2.83, 2.83 (m)			
3	31.92 - CH_2	2.70, 2.83 (m)			1.93
4	87.63 – C				2.70, 2.83, 3.66
5	52.14 – CH	3.66 dd (*J* 10.77, 2.75)	2.33	2.33, 1.93	2.33, 2.83
6	28.74 - CH_2	2.33, 1.93 (m)	3.66, 1.68, 1.53	1.53, 3.66,	1.93, 2.70, 2.83, 3.66
7	28.48 - CH_2	1.68, 1.53 (m)	1.22, 1.93, 2.33	2.33, 1.93	
8	29.39 - CH_2	1.14, 1.22 (m)		1.14, 1.07	
9	29.69 - CH_2	1.07, 1.07 (m)			
10	29.69 - CH_2	1.07, 1.22 (m)			
11	29.68 - CH_2	1.07, 1.14 (m)		1.07	
12	29.57 - CH_2	1.07, 1.14 (m)			
13	30.12 - CH_2	1.07, 1.14 (m)	1.07, 1.14		2.70, 2.70, 2.83, 3.66
14	22.76 - CH_2	1.07, 1.07 (m)	0.77, 1.07, 1.14	0.77, 1.07	
15	14.14 - CH_3	0.77 (3H, td, *J* 6.0, 2.3)		1.07	
16	174.52 – C				2.83, 3.66
17	175.12 – C				3.66

A literature search showed that this compound is known as spiculisporic acid. The first isolation was by Clutterbuck *et al* from *Penicillium spiculisporum* in 1931[6]. It was also isolated from *Penicillium crateriforme* in addition to succinic acid by Oxford et al [7]. Fujimoto *et al* also isolated spiculisporic acid from an ascomycetous fungus *Talaromyces panasenkoi* and they found it to be lethal to mice [8]. Yutaka *et al* investigated surface active properties of biosoap from spiculisporic acid to be used as biosurfactant [9].

The total synthesis of spiculisporic acid has been described by Goodwin [10]. He reported the synthesis of the two possible diastereomeric forms of spiculisporic acid shown in Figure 21.



(-)-Spiculisporic acid



(-)-Epi-Spiculisporic acid

Figure 21 The two diastereomers of spiculisporic acid

The two diastereomers showed some important differentiable IR, NMR spectral and optical rotation data. In the IR spectrum the epi-spiculisporic acid showed a lactone absorption at 1801 cm^{-1}, while the other isomer showed two absorptions at 1793 and 1778 cm^{-1} for the lactone ring. The other important differentiable spectroscopic data was in the ^1H NMR data. The epi-spiculisporic acid showed H-5 at δ_H 3.03 as a doublet of doublets (1H, J = 9.3, 4.2 Hz), whilst the other isomer showed the same proton at δ_H 3.01 also as a doublet of doublets (1H, J = 10.8, 2.7 Hz). The reported optical rotation of the synthesised spiculisporic acid was $[\alpha]_D^{25}$ – 10.2° (c 0.43, EtOH) and $[\alpha]_D^{25}$ – 6.3° (c 0.75, EtOH) for the Epi isomer. The natural product optical rotation was $[\alpha]_D^{20}$ – 15° (EtOH) [7].

By comparing the above spectroscopic data with that from the spiculisporic acid isolated from A311R endophytic fungi, it can be confirmed that the isolated acid is not the epi-isomer.

Spiculisporic acid was also studied as a Biosurfactant-based ultrafiltration system to remove metal ions (copper, zinc, cadmium, and nickel) from metal ion mixtures as well as a single metal component solution [11].

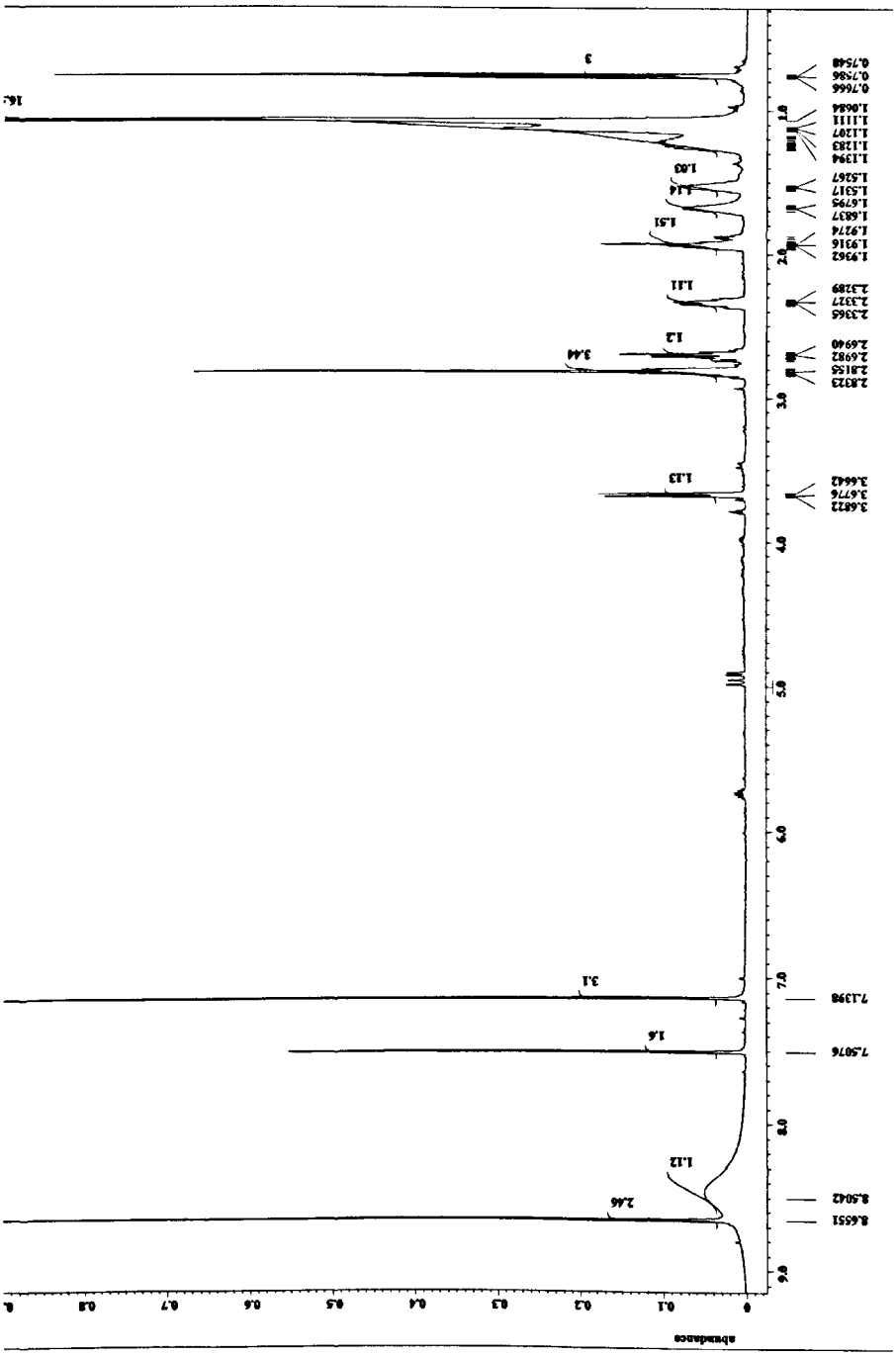


Figure 22 ^{1}H NMR spectrum (C_5D_5N) of 2.2

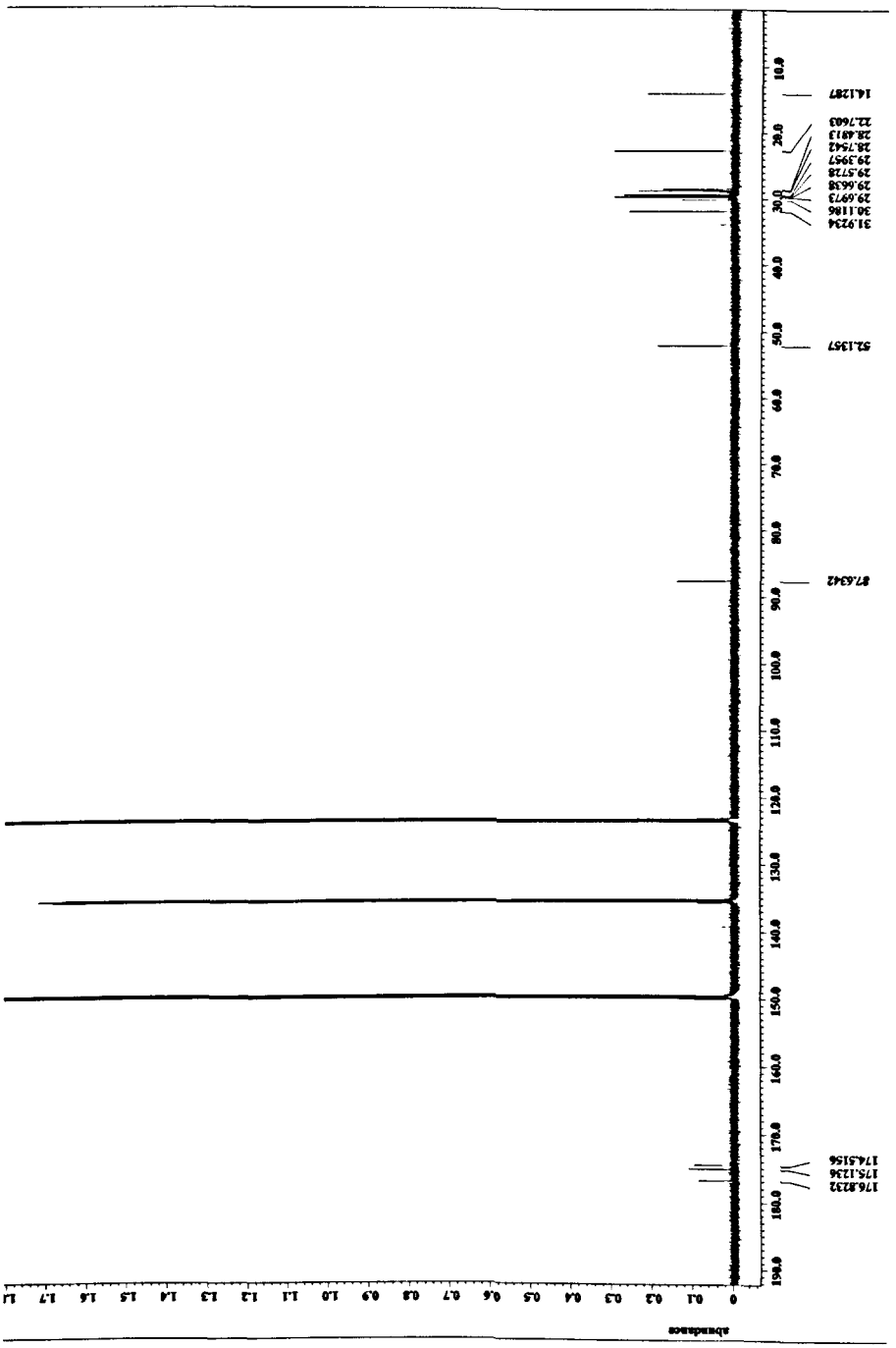


Figure 23 ^{13}C NMR spectrum (C_5D_5N) of 2.2

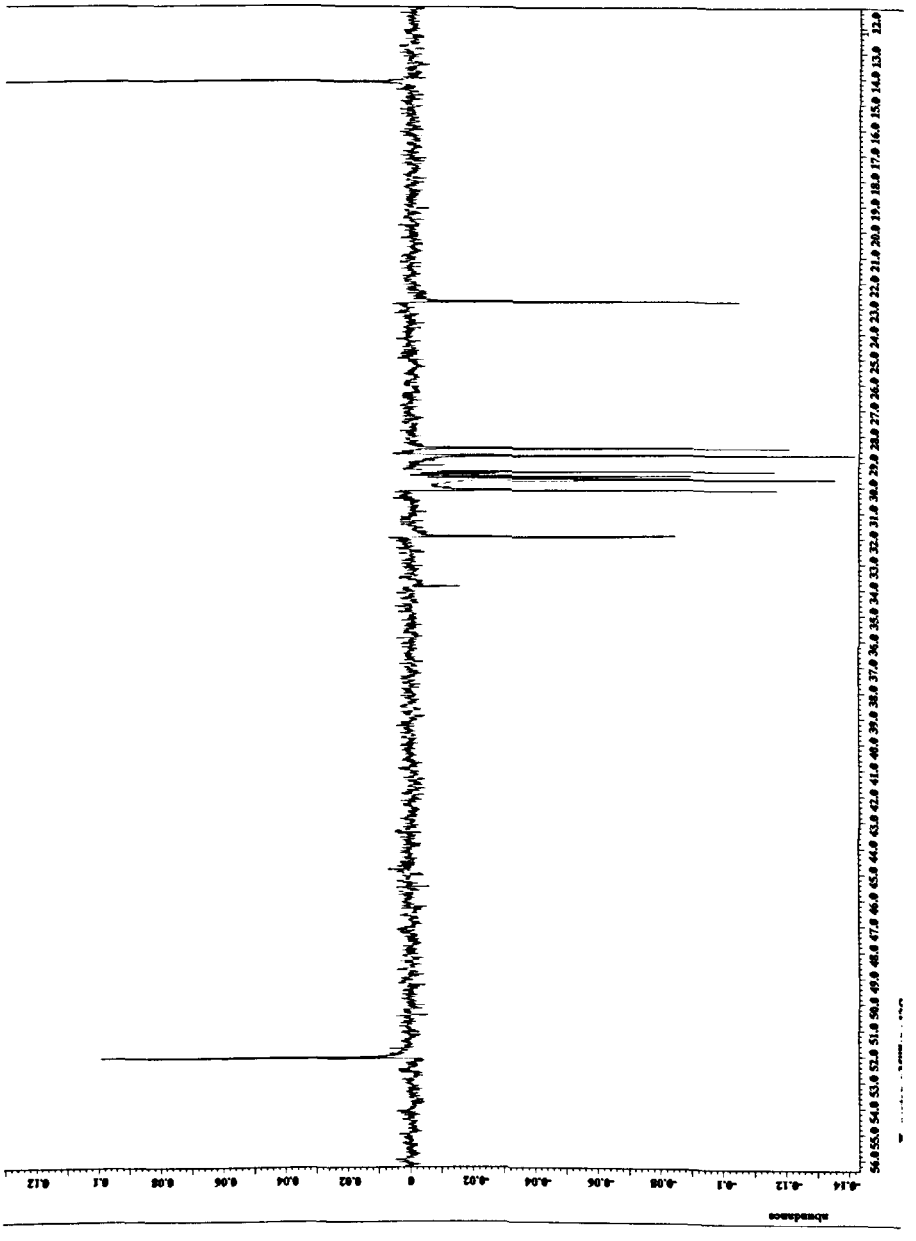


Figure 24 DEPT135 NMR spectrum of 2.2

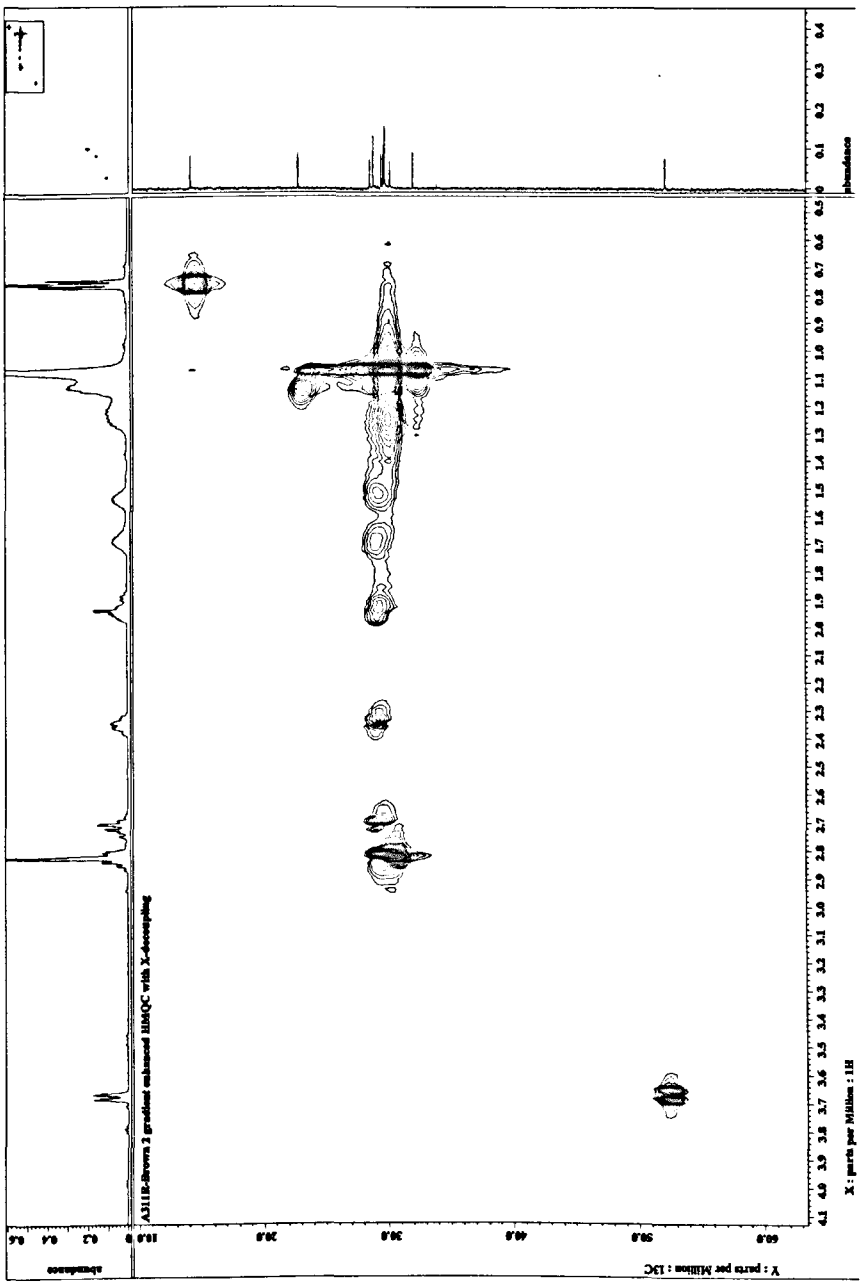


Figure 25 HMQC NMR spectrum of 2.2

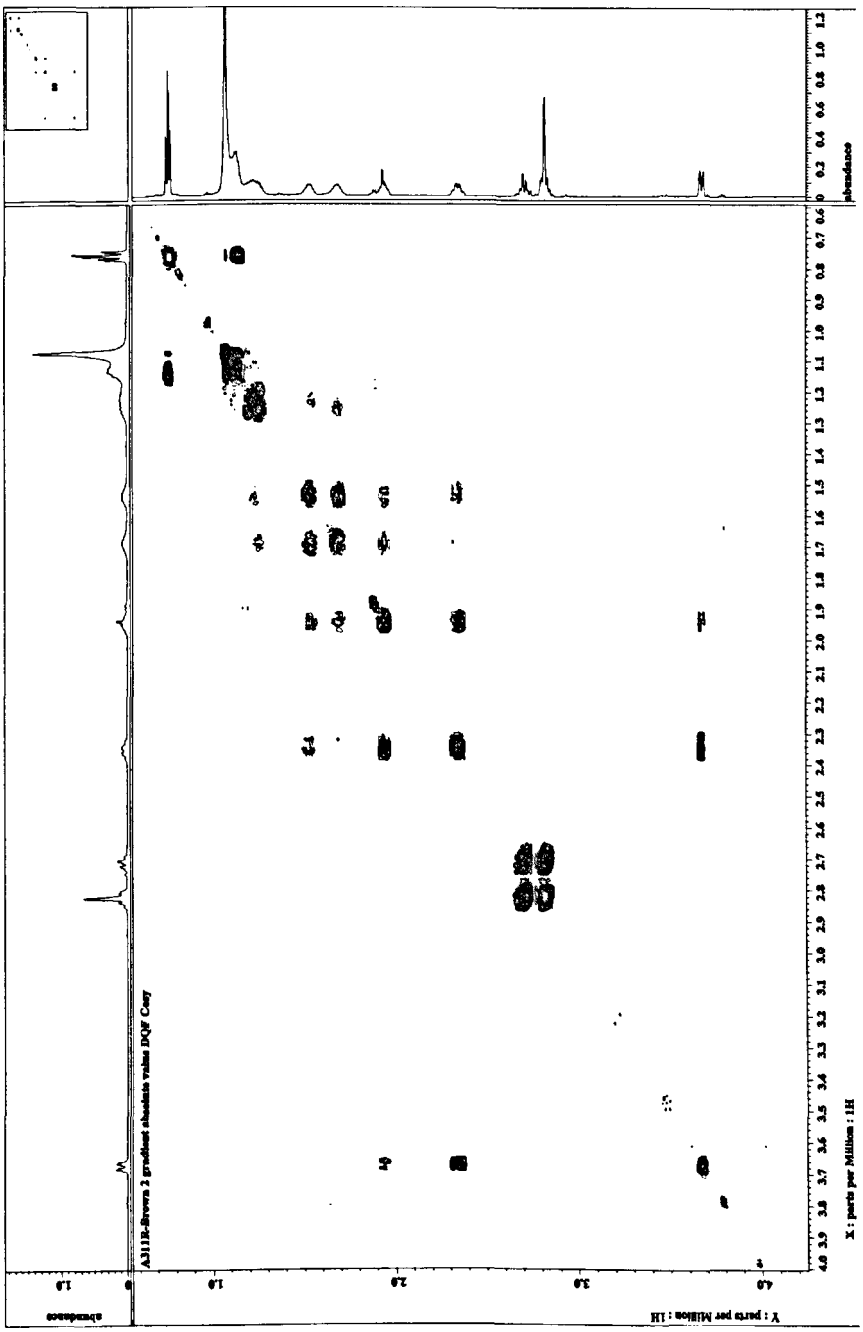


Figure 26 ^{1}H-^{1}H NMR COSY spectrum of 2.2

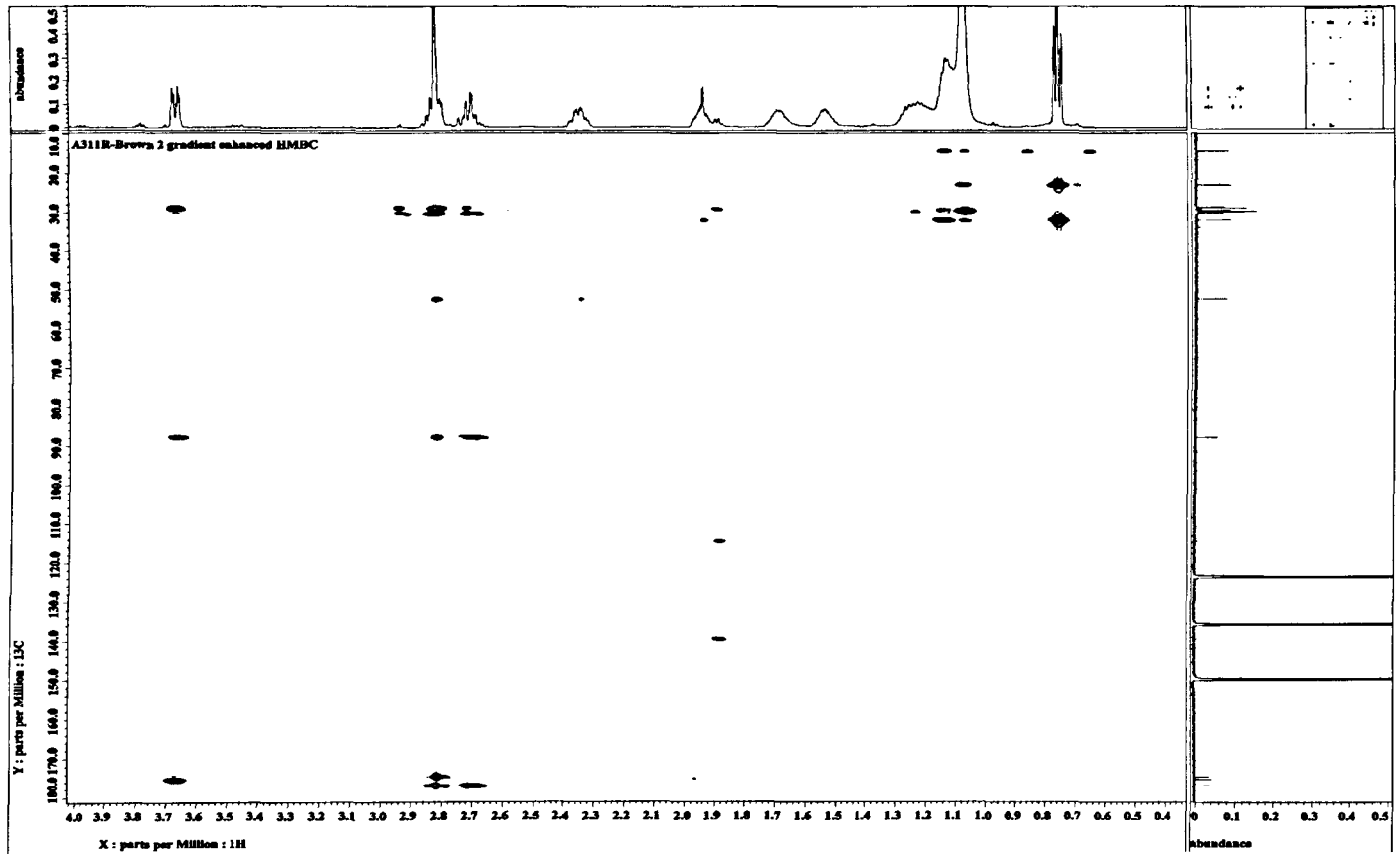


Figure 27 HMBC spectrum of 2.2

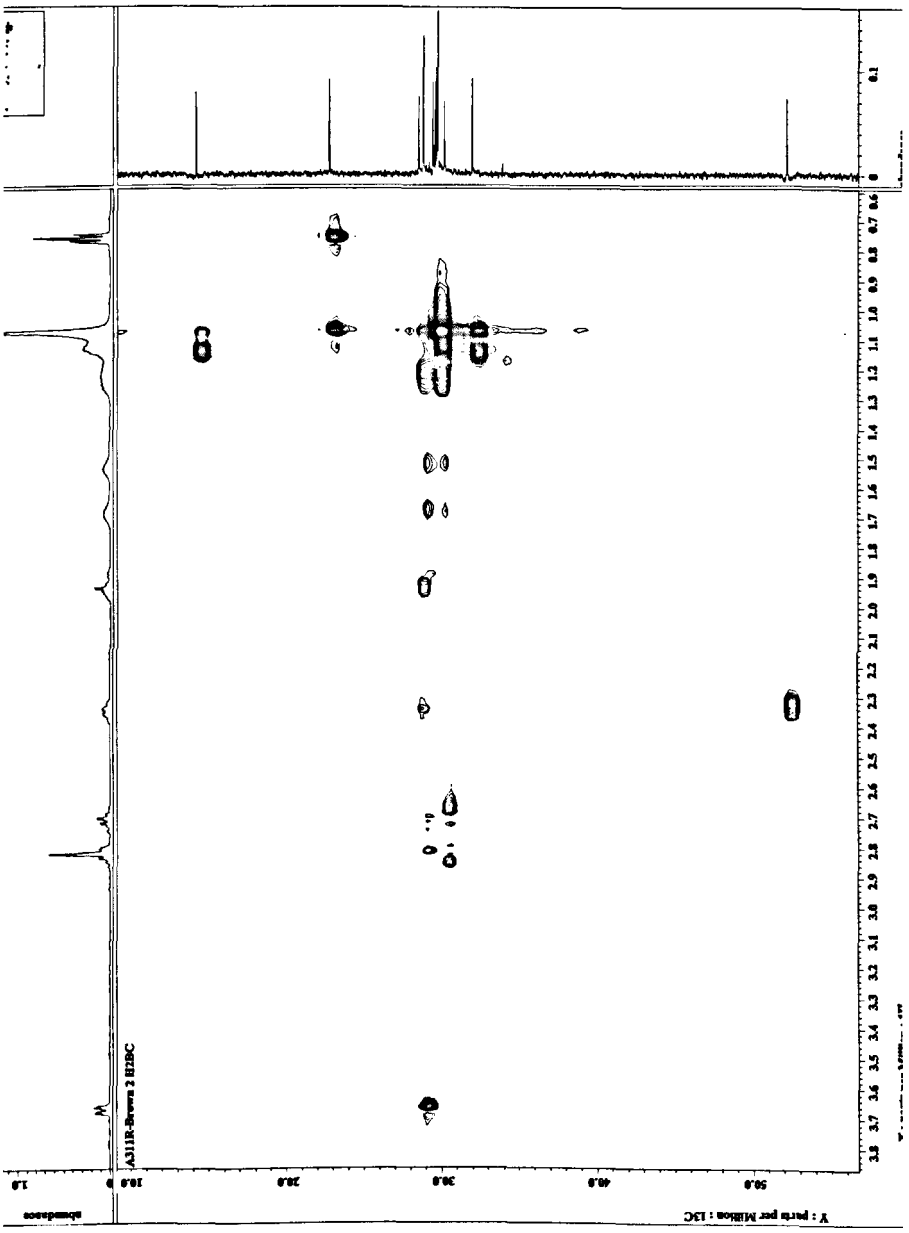


Figure 28 H2BC NMR spectrum of 2.2

2.2.1 Formation of dimethyl ester of spiculisporic acid

Methylation of spiculisporic acid **2.2** with diazomethane resulted in the formation of a dimethyl ester **2.2A**. The latter, a waxy material, gave a weak molecular ion in the ESI mass spectrum at 355 and a strong signal for $[M - CH_3]^-$ at m/z 341. The IR spectrum of **2.2A** showed absorptions at 1740 and 1714 cm^{-1} for ester carbonyl stretching and at 1764 cm^{-1} for the 5 ring lactone system. The weak broad absorption at 2550 cm^{-1}, which was seen in the IR spectrum of **2.2,** is absent.

Comparison of the NMR spectral data of **2.2** and **2.2A** proves the presence of two carboxylic acid groups in **2.2**. The ^1H NMR spectrum of **2.2A** (Figure 30) showed two extra methyl groups at δ_H 3.63, and 3.67, which in turn showed cross-peaks with δ_C 51.85 and 51.88 in the HMQC NMR spectrum, due to the formation of two methoxy groups when **2.2** reacts with diazomethane. Table 6 shows the ^1H and ^{13}C NMR data of both **2.2** and **2.2A**.

The ^{13}C NMR spectrum of **2.2A** (Figure 31) showed three carbonyls at δ_C 171.53, 172.54 and 175.68. The first two are shifted by 3 ppm to higher field compared to their corresponding carbonyls in **2.2** while the one at δ_C 175.68 does not show this known shift that occurs when a carboxylic carbonyl group is methylated. This is evidence that this carbonyl group wasn't a carboxylic acid type and that compound **2.2** contains only two carboxylic acids plus a lactone carbonyl group as shown by Figure 29 . After esterification the IR spectrum showed main absorptions at 1740 and 1764 cm^{-1} with the disappearances of the carboxylic hydrogen bonding absorption at 2550 cm^{-1}.

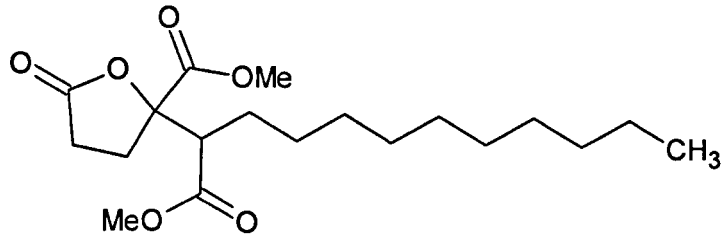


Figure 29 Dimethyl ester of spiculisporic acid 2.2A

Table 6 ^{1}H and ^{13}C NMR data of both 2.2 and 2.2A.

2.2			2.2.A		
#	δ_C	δ_H	#	δ_C	δ_H
1	176.82 – C		**1**	175.68 – COO Me	
2	28.75 - CH_2	2.83, 2.83 (m)	**2**	27.99 - CH_2	1.18, 1.11 (m)
3	31.92 - CH_2	2.70, 2.34 (m)	**3**	31.94 - CH_2	2.67, 2.58 (m)
4	87.63 – C		**4**	86.52 – C	
5	52.14 – CH	3.66 (dd)	**5**	52.88 – CH	3.26 (dd)
6	28.74 - CH_2	1.68, 1.53 (m)	**6**	27.95 - CH_2	1.65, 1.34 (m)
7	28.48 - CH_2	2.33, 1.93 (m)	**7**	27.92 - CH_2	1.26, 1.18 (m)
8	29. 39 - CH_2	1.14, 1.22 (m)	**8**	29. 37- CH_2	1.11, 1.11 (m)
9	29.69 - CH_2	1.07, 1.07 (m)	**9**	29.56 - CH_2	1.11, 1.11 (m)
10	29.69 - CH_2	1.07, 1.22 (m)	**10**	29.64 - CH_2	1.11, 1.18 (m)
11	29.68 - CH_2	1.07, 1.14 (m)	**11**	29.43 - CH_2	1.11, 1.18 (m)
12	29.57 - CH_2	1.07, 1.14 (m)	**12**	29.40 - CH_2	2.56, 2.56 (m)
13	30.12 - CH_2	1.07, 1.14 (m)	**13**	29.64 - CH_2	1.11, 1.11 (m)
14	22.76 - CH_2	1.07, 1.07 (m)	**14**	22.77 - CH_2	1.11, 1.11 (m)
15	14.14 - CH_3	0.77 (td)	**15**	14.13 - CH_3	0.78 (td)
16	174.52 – C		**16**	171.53 – **C**	
17	175.12 – C		**17**	172.54 – **C**	3.67 (s)
			18	51.85 - OMe	
			19	51.88 - OMe	3.63 (s)

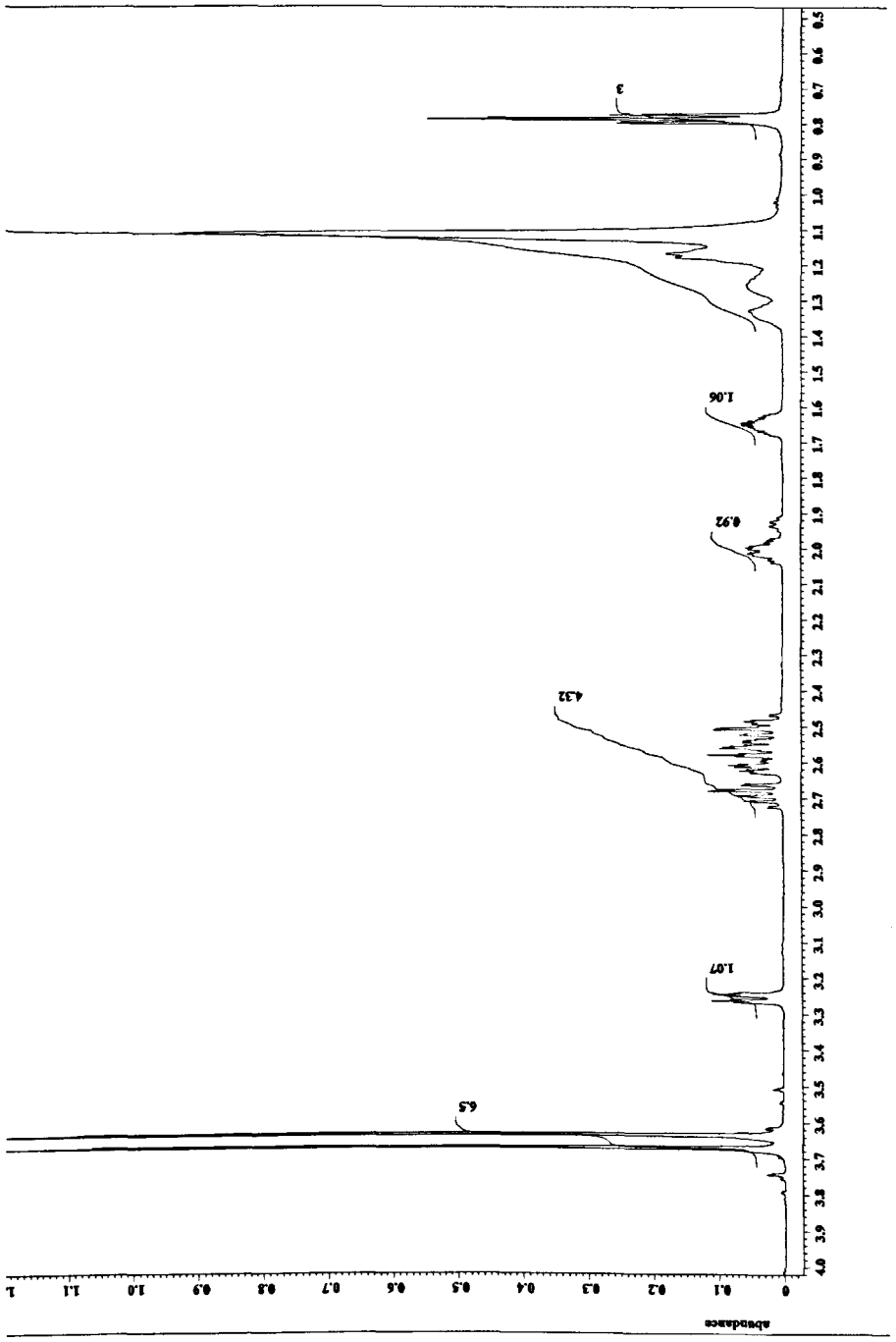


Figure 30 ^{1}H NMR spectrum (C_5D_5N) of 2.2A

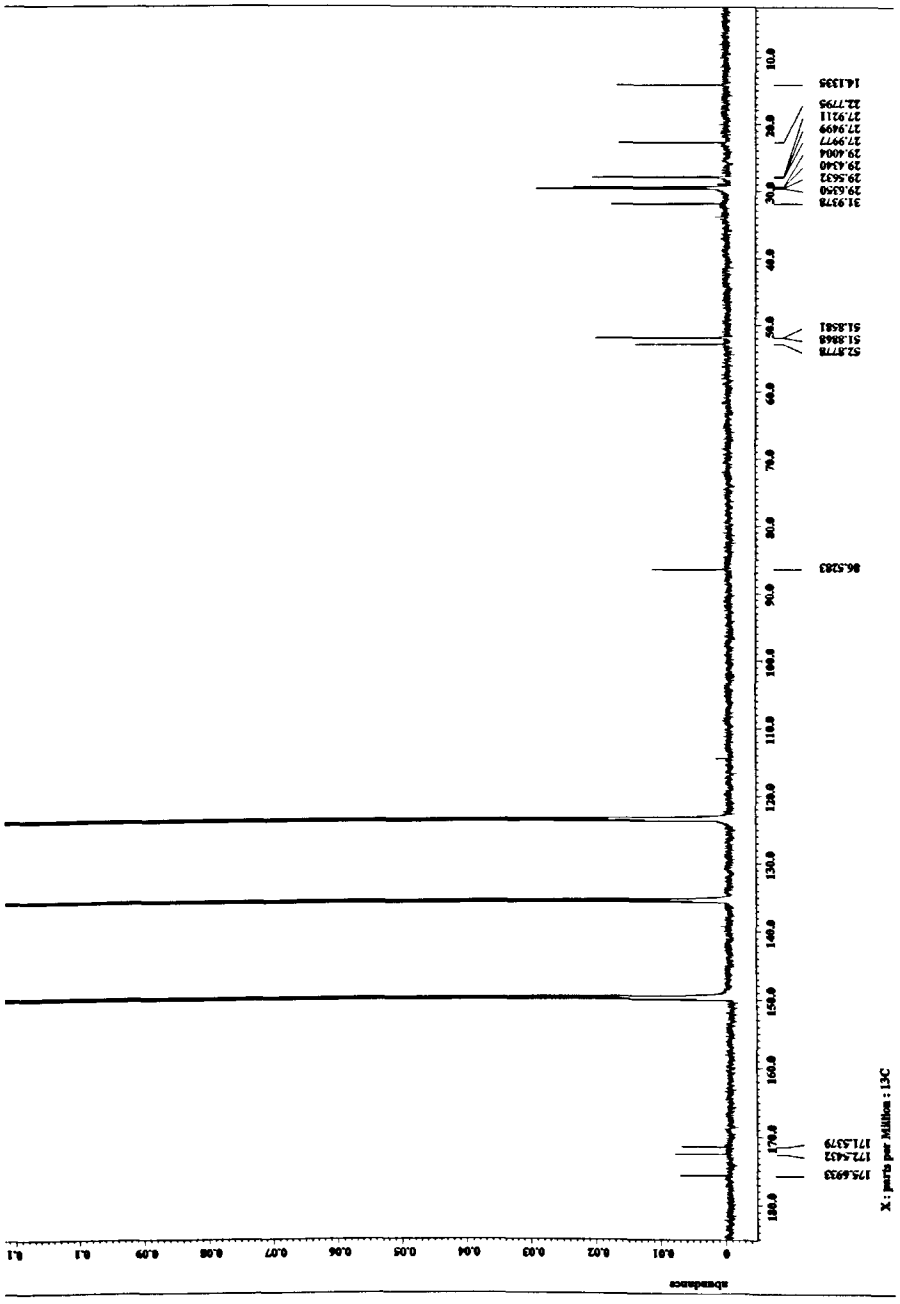


Figure 31 ^{13}C NMR spectrum (C_5D_5N) of 2.2A

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Chapter 3: Secondary metabolites from X.6RD12

3.0 6RD12 fungus overview

The fungus coded 6RD12 was isolated from palm trees in Thailand. It was received in Petri dishes as shown in Figure 32. The front view of the Petri dish showed white mycelium with black circular areas. The reverse side of the fungus was orange.

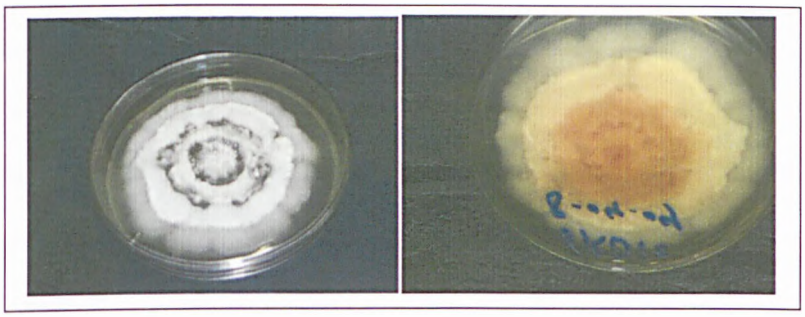


Figure 32 Fungi 6RD12 as received from Thailand

The fungus was sub-cultured into master tubes and 5 conical flasks (250 ml) containing the aqueous malt extract-glucose mixture. The conical flasks were kept under observation for 3 weeks (Figure 33).

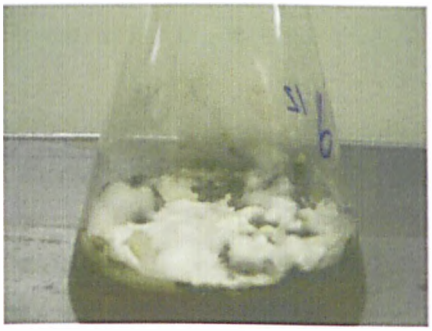


Figure 33 Fungus 6RD12 in flask after 3 weeks

The fungus developed black cylindrical stromata with white tips after 2 weeks. The fungus was then cultured in Thompson bottles for 8 weeks. The fungus during the first 4 weeks grew as white mycelium and then developed black areas.

The mature cultures were harvested and the mycelium recovered by filtration through a muslin cloth. The aqueous filtrate was extracted with ethyl acetate in a 5.0 L separating funnel. The ethyl acetate fraction was dried over anhydrous sodium sulphate. After removal of the solvent 3.35 g of crude extract was obtained.

3.1 Isolation and structure elucidation of 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1H-isochromen-8(5H)-one (3.1)

The normal phase TLC analysis of the ethyl acetate extract eluted with toluene, ethyl acetate and acetic acid (50:49:1) showed only a dark brown spot at the origin of the TLC. As a result, a reverse phase TLC was used, which showed 3 weak UV active spots at R_f 0.96, 0.85 and 0.46. Sephadex LH-20 (70 g) in a chromatography column (50 × 2 cm) was used to separate the components of the ethyl acetate crude extract (3 g).

Methanol-water (80:20) mobile system was used to elute the column. The first compound (**3.1**) collected was white solid (95 mg), mp 180-185 °C, HRESIMS $[M+H]^+$ *m/z* 243.12274; $[\alpha]_D^{22}$ - 160° (c 0.5, MeOH); $IR_{(ATR)}$ ν_{max} cm^{-1} : 3378, 1673 and 1105. The main IR absorptions suggested presence of hydroxyl and carbonyl groups.

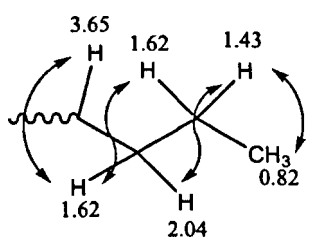
HREIMS analysis of (**3.1**) gave a molecular ion at $[M+H]^+$ m/z 243.12274, which gives a molecular formula of $C_{12}H_{18}O_5$. The ^1H NMR spectrum (Figure 36) run in (C_5D_5N) revealed one methyl at δ_H 0.82 (3H, t, *J* 7.4 Hz), and methylene protons at δ_H 1.43 (1H, m), 1.62, (2H, m), 2.04 (1H, m), 2.81 (1H, dd, *J* 3.6, 15.81Hz), 3.21

(1H, dd, *J* 8.1,15.81), 4.77 (1H, m) and 4.42 (1H, dt, *J* 16.3, 2.4); methine protons at δ_H 3.65 (1H, td, *J* 2.75, 8.1 Hz), 4.75 (1H, m), 5.42 (1H, bs) and 4.51 (1H, m).

The ^{13}C and DEPT 135 NMR spectra (Figure 37 and Figure 38, C_5D_5N) of compound **3.1** revealed 12 carbons: one methyl at δ_C 14.17, four methylene at δ_C 18.98, 34.91, 43.50 and 63.48, four methine δ_C 66.48, 66.82, 69.59, 79.08 and three quaternary carbons δ_C 132.60, 155.27 and 197.08. The prominence of the M-18 peak (m/z 225 and 207) in the EIMS spectra of **3.1** suggested the presence of hydroxyl groups. The molecular formula suggested a total of four degrees of unsaturation.

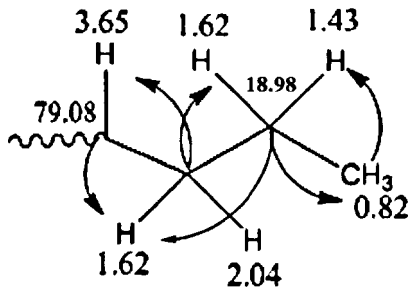
The ^{13}C, ^1H, HMQC, DEPT135, ^1H-^1H Cosy, HMBC and H2BC NMR spectra allowed the assignment of all the protons and their connectivities and identification of the associated carbon signals.

Evaluation of the 2D DQF-COSY spectrum (Figure 39) allowed the identification of the spin systems. The methyl protons at δ_H 0.82 are coupled to the methylene protons at δ_H 1.42 and 1.62 [showed cross peak with δ_C 18.98 in the HMQC spectrum (Figure 40)], which were in turn coupled to the methine proton at δ_H 3.65. This sequence of the proton coupling led to the establishment of a partial sub-structure **3.1a**.



Partial structure **3.1a** showing ^1H-^1H connectivities

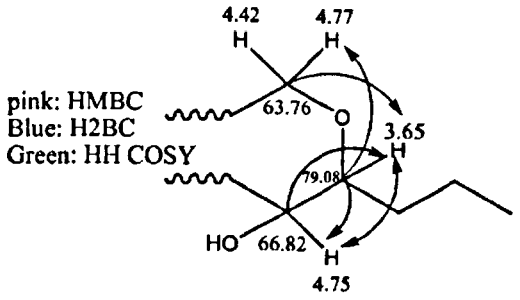
This spin system was refined further through interpretation of a H2BC spectrum (Figure 42), which can be described as follows; the methylene carbon observed at δ_C 18.98 correlates to the methyl proton at δ_H 0.82 and to the methylene protons at δ_H 1.62. The last in turn correlates to the methine carbon at δ_C 79.08.



Partial structure **3.1a** showing H2BC correlations (Blue arrows)

This partial structure was also supported by the HMBC experiment (Figure 41), which showed correlations between the proton at δ_H 3.65 and the carbon at δ_C 18.98. The chemical shift of δ_C 79.08 suggests a carbon attached to oxygen. The H2BC spectrum showed correlation between δ_C 79.08 and δ_H 4.75, which has a cross peak with δ_C 66.82 (good indication of a secondary alcohol) in the HMQC spectrum confirming that those two carbons are neighbours to each other. Furthermore, the presence of coupling between the two protons at δ_H 3.65 and 4.75 confirms the neighbourhood of these atoms.

Further HMBC correlations were observed between δ_C 79.08 and δ_H 4.77, which has a cross peak with δ_C 63.76 in the HMQC spectrum. This carbon in turn showed an HMBC correlation with the methine proton at δ_H 3.65. This information led to the sub-structure **3.1b** as shown below.



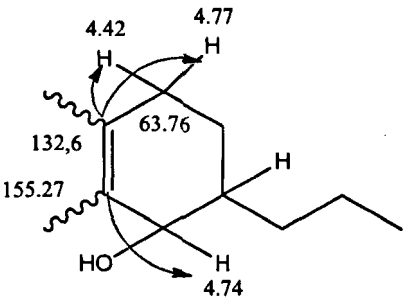
3.1b

Table 7 summarises 1D and 2D NMR data (C_5D_5N)

Table 7 1D and 2D NMR data of 3.1 (C_5D_5N).

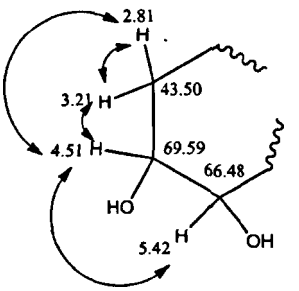
No	δ_C	δ_H	HMBC	H-H COSY	H2BC
1	14.17 – CH_3	0.82 (t, *J* 7.4)	2 (1.43)	2	2
2	18.98 – CH_2	1.62, 1.43 (m)	1, 9	3, 3, 1	1
3	34.91 – CH_2	2.04, 1.62 (m)	1, 2 (1.43)	9, 2	2, 2, 9
4	43.50 – CH_2	2.81 (dd, *J* 3.6, 12.18)		4, 8	8
		3.21 (dd, *J* 8.1, 7.74)			
5	63.76 – CH_2	4.42 (dt, *J* 16.3, 2.4)	9	5	
		4.77 (m)			
6	66.48 – CH	5.42 (bs)	4, 4	8	
7	66.82 – CH	4.75 (m)	9	9	9, 6
8	69.59 – CH	4.51 (m)	4	4, 4, 6	6, 4
9	79.08 – CH	3.65 (td, *J* 2.75, 8.1)	7	7, 3	3, 7
10	132.60 – C		5, 5		
11	155.27 – C		7, 8		
12	197.08 – C		4, 4		

The observation of more HMBC correlations between the methylene protons resonating at δ_H 4.77 and 4.42, which are connected to δ_C 63.76 and the carbon signal at δ_C 132.60 allowed the placement of the double bond within the sub-structure **3.1b**. The presence of an HMBC correlation between the proton at δ_H 4.75 and the carbon signal at δ_C 155.27 allowed for closure of the six ring to give sub-structure **3.1c**.



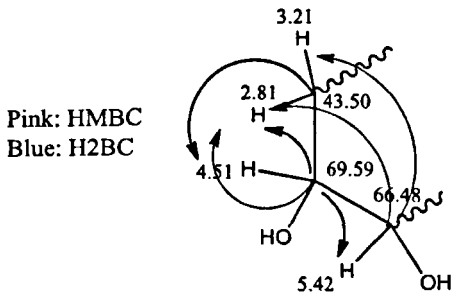
3.1c

Another sub-structure unit was established by tracking the rest of the correlations in the ^{1}H-^{1}H COSY and HMBC spectra. In the ^{1}H-^{1}H COSY the methylene protons at δ_H 2.81 and 3.21, which show a cross peak with δ_C 43.50, both showing correlation to δ_H 4.51, which shows a cross peak at δ_C 69.59, which in turn couples to δ_H 5.42 to give the partial sub-structure **3.1d**.



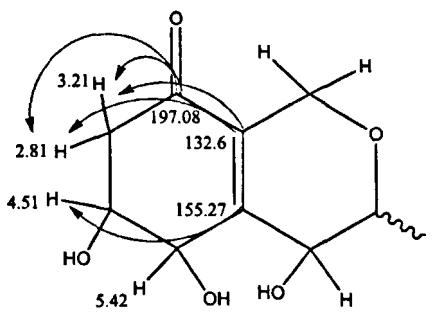
3.1d

The spin system **3.1d** was further confirmed through interpretation of the HMBC and H2BC NMR data. In the HMBC spectrum, the protons at δ_H 2.81 and 3.21 showed correlations with the carbon at δ_C 66.48. The same protons showed H2BC correlations with the δ_C 69.59, which showed another correlation to the proton at δ_H 5.42 confirming the positions of the neighbouring atoms as shown by partial structure **3.1e**.



3.1e

More HMBC correlations were observed between the protons at δ_H 2.81 and 3.21 and the carbon at δ_C 197.08 allowed the addition of a carbonyl group to the above substructure. The HMBC correlation between the proton at δ_H 4.51, which is connected to δ_C 69.59, and the double bond carbon at δ_C 155.27 helped to connect sub-structure **3.1c** with sub-structure **3.1e** to give **3.1f**:



3.1f

So the final structure of compound **3.1** is suggested to be as shown by Figure 34.

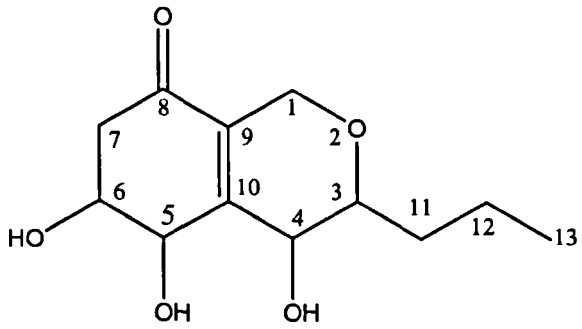


Figure 34 Structure of 3.1

A Scifinder search revealed that compound **3.1**, 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one has not been reported previously either from a synthetic or a natural product source. Table 8 summarises the NMR data according to Figure 34.

Table 8 NMR data (C_5D_5N) of 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one (3.1)

No	δ_C	δ_H
1	63.76 – CH_2	4.42 (dt, *J* 16.3, 2.4), 4.77 (m)
3	79.08 – CH	3.65 (td, *J* 2.75, 8.1)
4	66.82 – CH	4.75 (m)
5	66.48 – CH	5.42 (bs)
6	69.59 – CH	4.51 (m)
7	43.50 – CH_2	2.81 (dd, *J* 3.6, 15.81)
		3.21 (dd, *J* 8.1, 15.81)
8	197.08 – C	
9	132.60 – C	
10	155.27 – C	
11	34.91 – CH_2	2.04, 1.62 (m)
12	18.98 – CH_2	1.62, 1.43 (m)
13	14.17 – CH_3	0.82 (t, *J* 7.4)

3.1.1 Stereochemistry of 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1H-isochromen-8(5H)-one (3.1)

Having established the structure of compound **3.1**, the next challenge was to resolve the stereochemical relationships of the three hydroxyl groups and the *n*-propyl side chain. This proved a particular challenge. Ideally the way forward was to use XRD, but unfortunately a suitable crystal could not be grown.

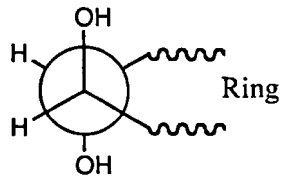
It did not prove feasible to analyse the ^{1}H NMR spectrum of **3.1** in C_5D_5N to determine coupling constants, which could be used in conjunction with the Karplus Equation [1], to determine dihedral angles between adjacent substituents. Most of the signals of interest in the δ_H 3.5 to 5.5 region of the spectrum are poorly resolved due to coupling between most of the protons of interest and the protons of the hydroxyl groups (Figure 36).

This problem was resolved by adding a drop of deuterium oxide to the sample in C_5D_5N and re-acquiring the ^{1}H NMR data with the probe temperature increased to 30 °C. This resulted in a spectrum with much improved resolution in the δ_H 3.5 to 4.8 region of the spectrum (Figure 43) and the appearance of signals with clearly defined multiplicities. The only down side was due to the HOD signal masking the signal at δ_H 5.42 from the methine proton on C-5. A complete analysis of the new ^{1}H NMR spectrum is presented in Table 9.

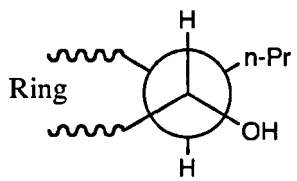
Table 9 ^{1}H NMR data for Compound 3.1 plus D_2O in C_5D_5N at 30 °C

Carbon No	δ_H
1	4.35 (1H, d, *J* 16.32 Hz)
	4.66 (1H, d, *J* 16.32 Hz)
3	3.61 (1H, dt, *J* 8.16, 2.58 Hz)
4	4.71 (1H, d, *J* 7.56 Hz)
5	5.38 (1H, bs, LHS edge HOD peak)
6	4.48 (1H, ddd, *J* 8.33, 3.61, 3.52 Hz)
7	3.17 (1H, dd, *J* 15.98, 8.42 Hz)
	2.76 (1H, dd, *J* 15.98, 3.69 Hz)
11	1.59 (1H, m)
	1.97 (1H, m)
12	1.39 (1H, m)
	1.53 (1H, m)
13	0.79 (3H, t, *J* 7.30 Hz)

The magnetically non-equivalent methylene protons on C-7 show well-defined doublet of doublet signals at δ_H 2.76 and 3.17 with *J* values of 15.98, 8.42 and 3.69 Hz. Clearly the 15.98 Hz coupling is due to geminal coupling of the methylene protons. The remaining *J* values relate to coupling to the methine proton on C-6 at δ_H 4.48. Examination of the *J* values associated with the latter gives 8.33, 3.61 and 3.52. The values of 8.33 and 3.61 essentially match with those of 8.42 and 3.69 associated with the protons on C-7. This leaves a coupling constant of 3.52 Hz for the coupling to the methine proton on C-5, which in turn means a dihedral angle between the protons on C-6 and C-5 of 60° typical of an equatorial- equatorial relationship, thus the OH groups must be in an *anti* relationship to each other.



Turning attention to the other ring, the methine proton on C-4 appears as a doublet at δ_H 4.71 with *J* coupling of 7.56 Hz to the methine proton on C-3. This suggests that the H-3, H-4 protons have an *anti* relationship and that the *n*-propyl and the hydroxyl groups are in an equatorial-equatorial relationship.



A series of phase sensitive 2D NOESY experiments with variations in the mixing time of 0.1s in the range 0.4 to 0.8s failed to detect any cross peak between the protons on H-4 and H-5 indicative of a *trans* relationship.

Putting all of this information together suggested that the 3-dimensional structure of compound **3.1** was as shown by Figure 35.

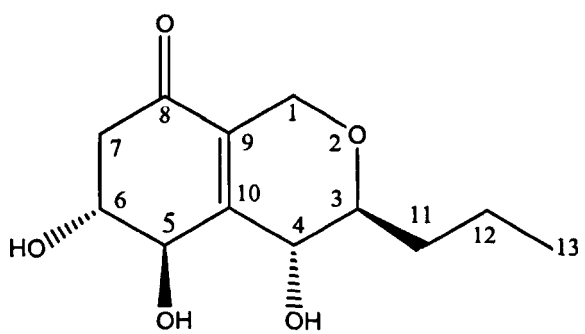


Figure 35 Relative stereochemistry of compound 3.1

3.1.2 Antiplasmodial test of 3.1

Compound **3.1** was tested for antiplasmodial activity against *Plasmodium falciparum* strain K1. The test was done in The Bradford School of Pharmacy, University of Bradford by the research group of Dr Colin W. Wright. It gave IC_{50} = 3.74 μg/mL (mean of two experiments).

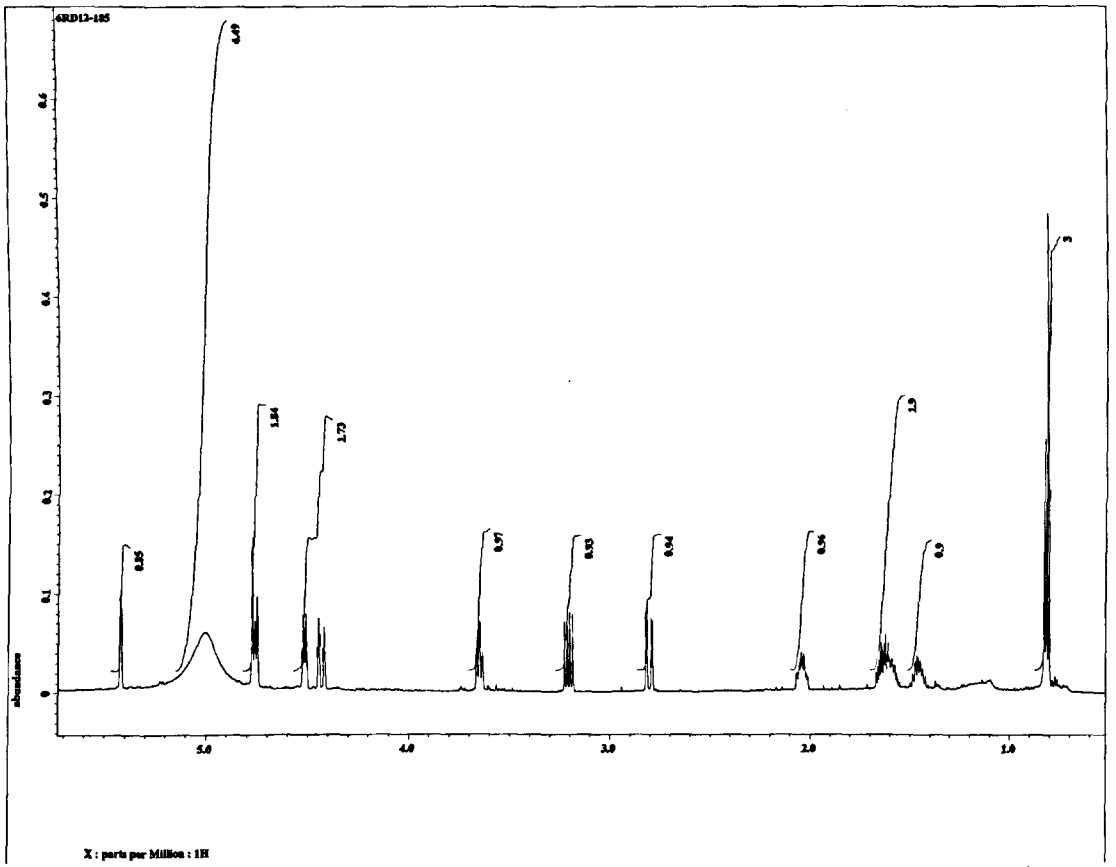


Figure 36 ^{1}H NMR spectrum (C_5D_5N) of 3.1

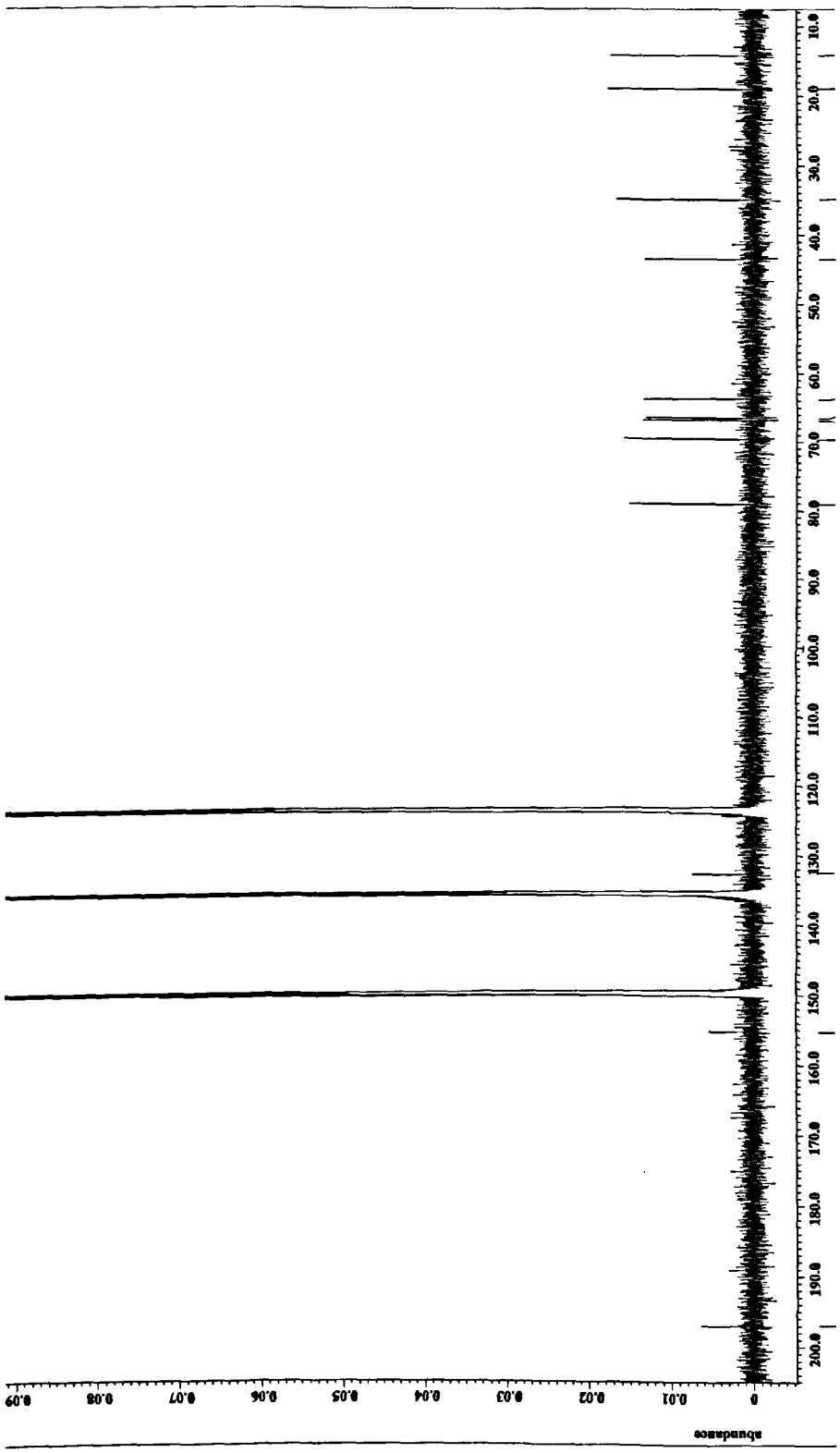


Figure 37 ^{13}C NMR spectrum (C_5D_5N) of **3.1**

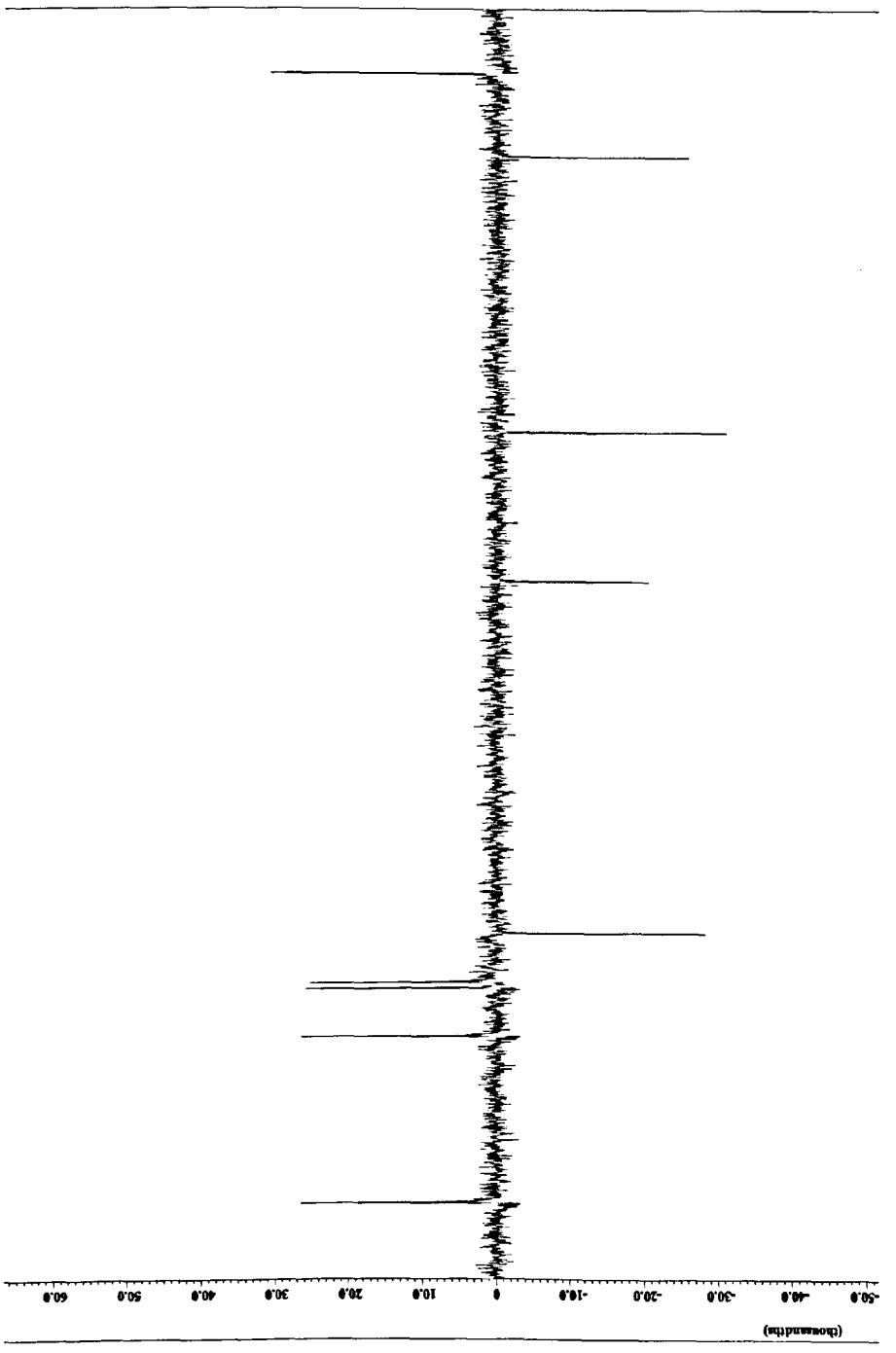


Figure 38 DEPT 135 spectrum of 3.1

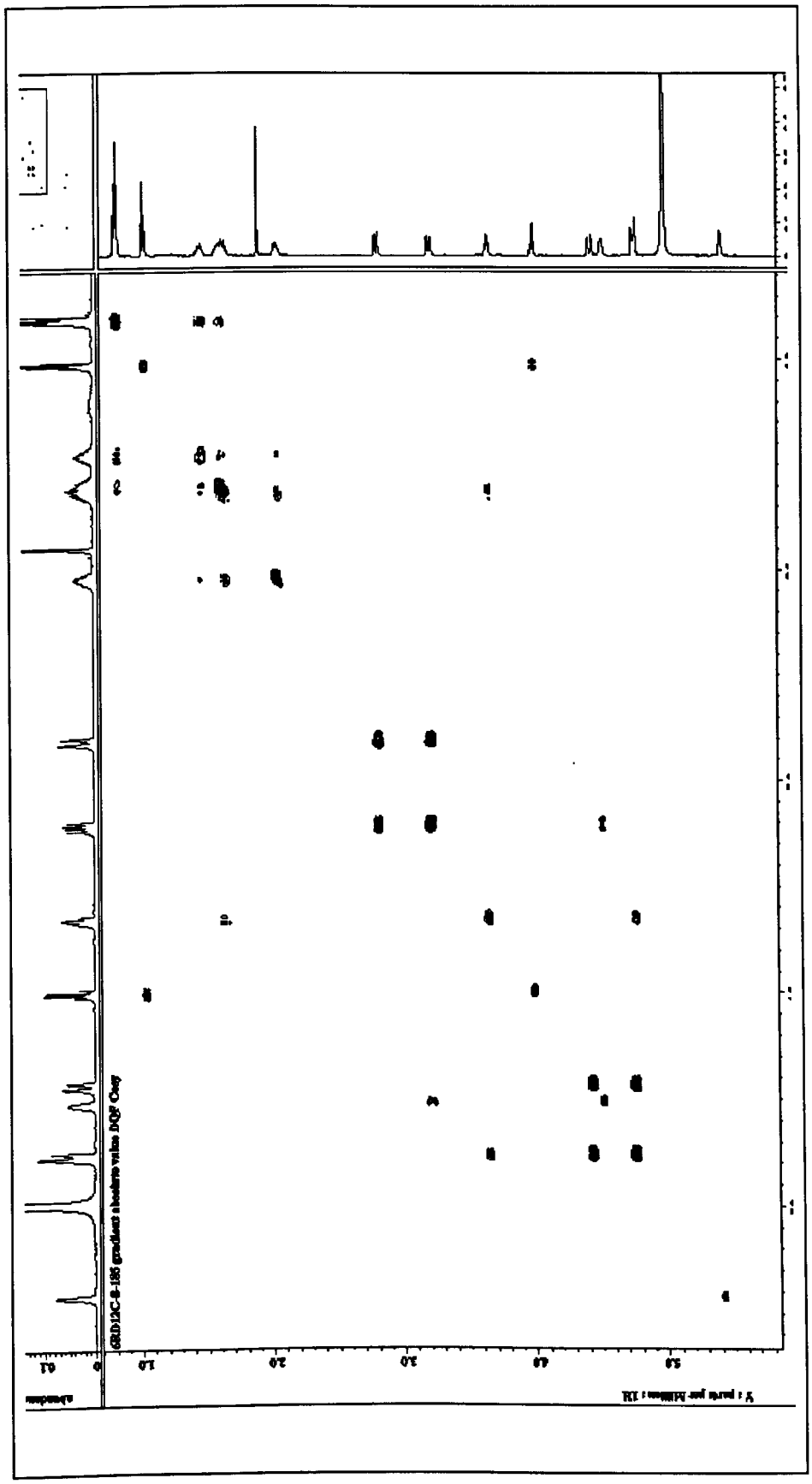


Figure 39 ^{1}H-^{1}H COSY NMR spectrum of 3.1

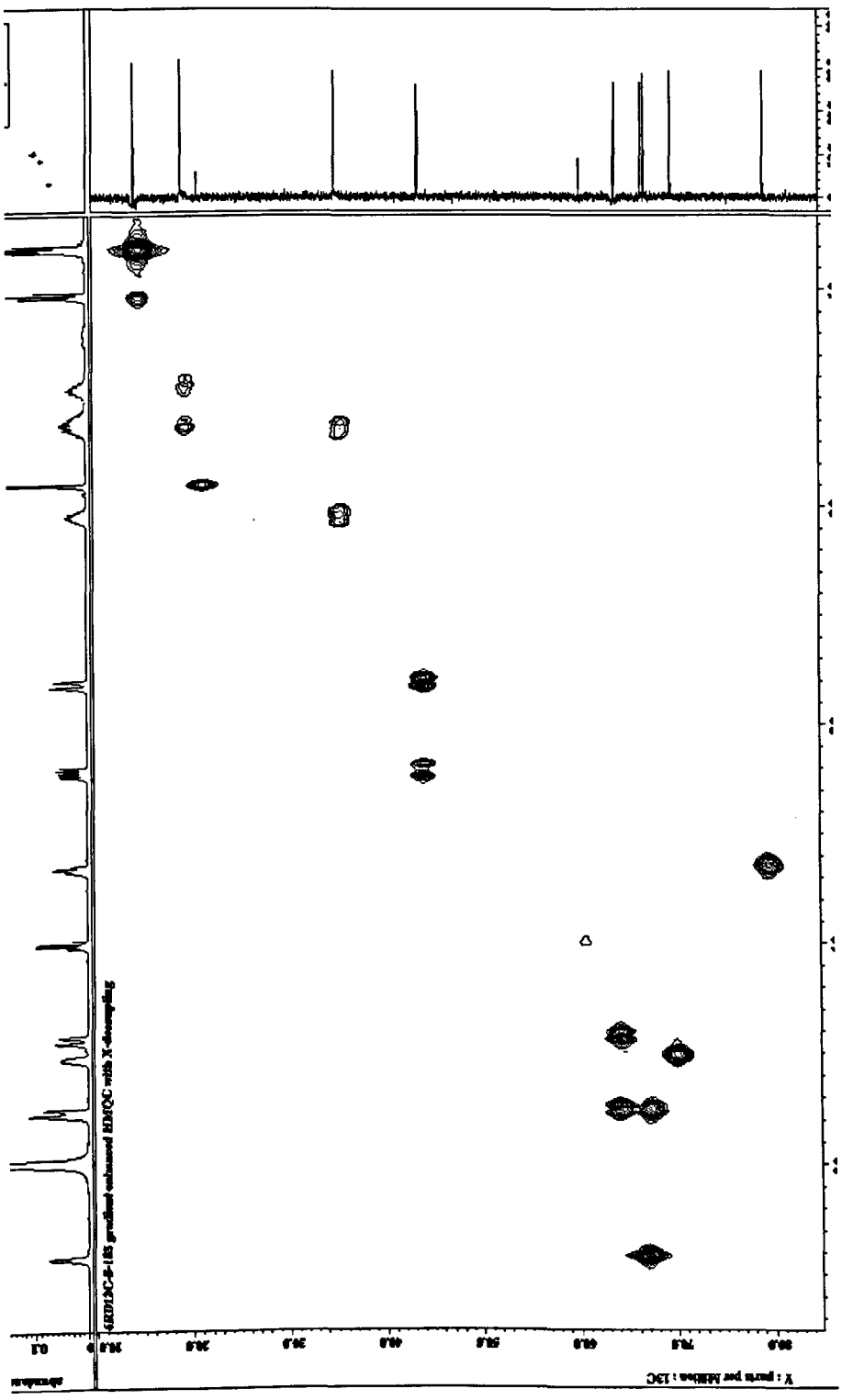


Figure 40 HMQC NMR spectrum of 3.1

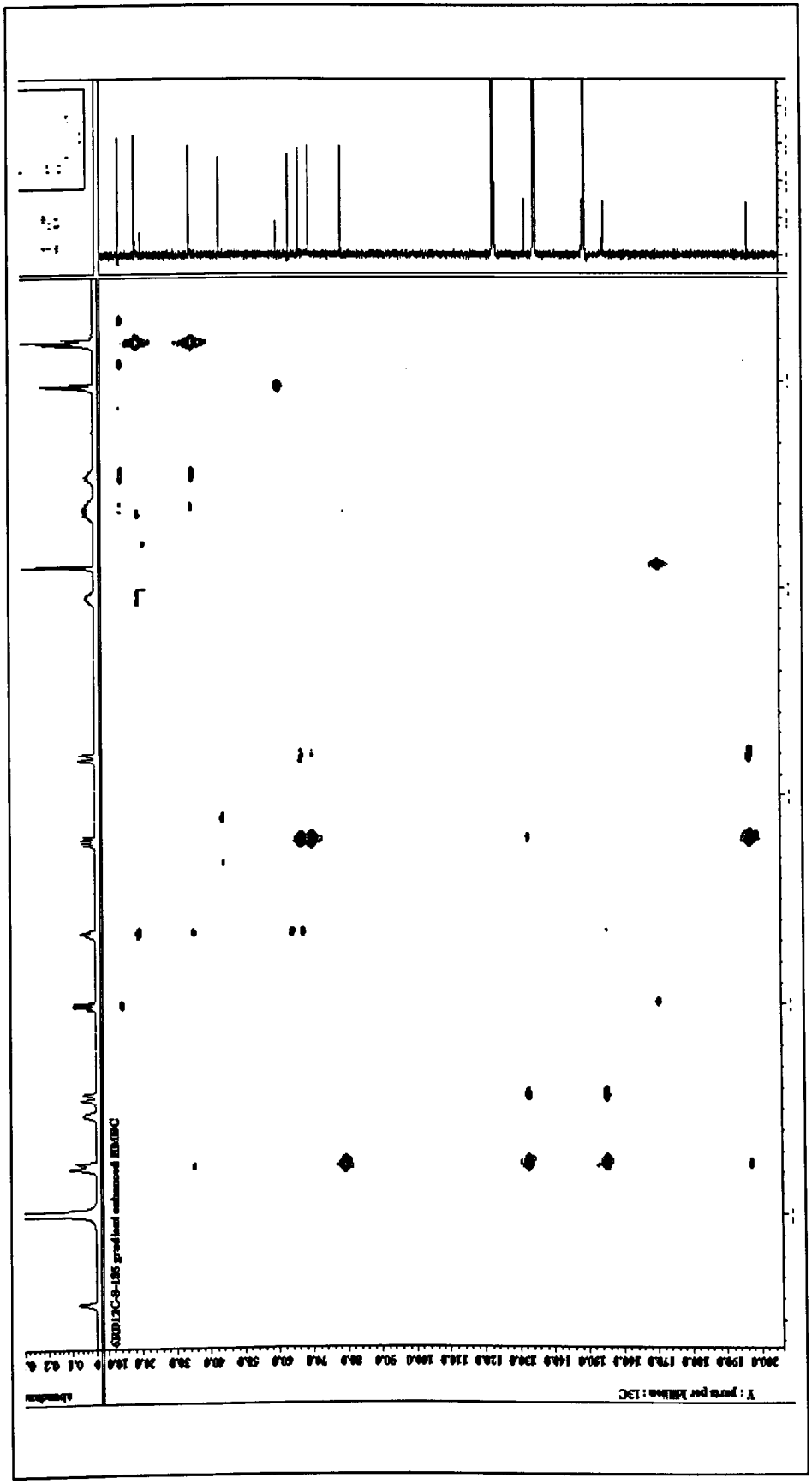


Figure 41 HMBC NMR spectrum of 3.1

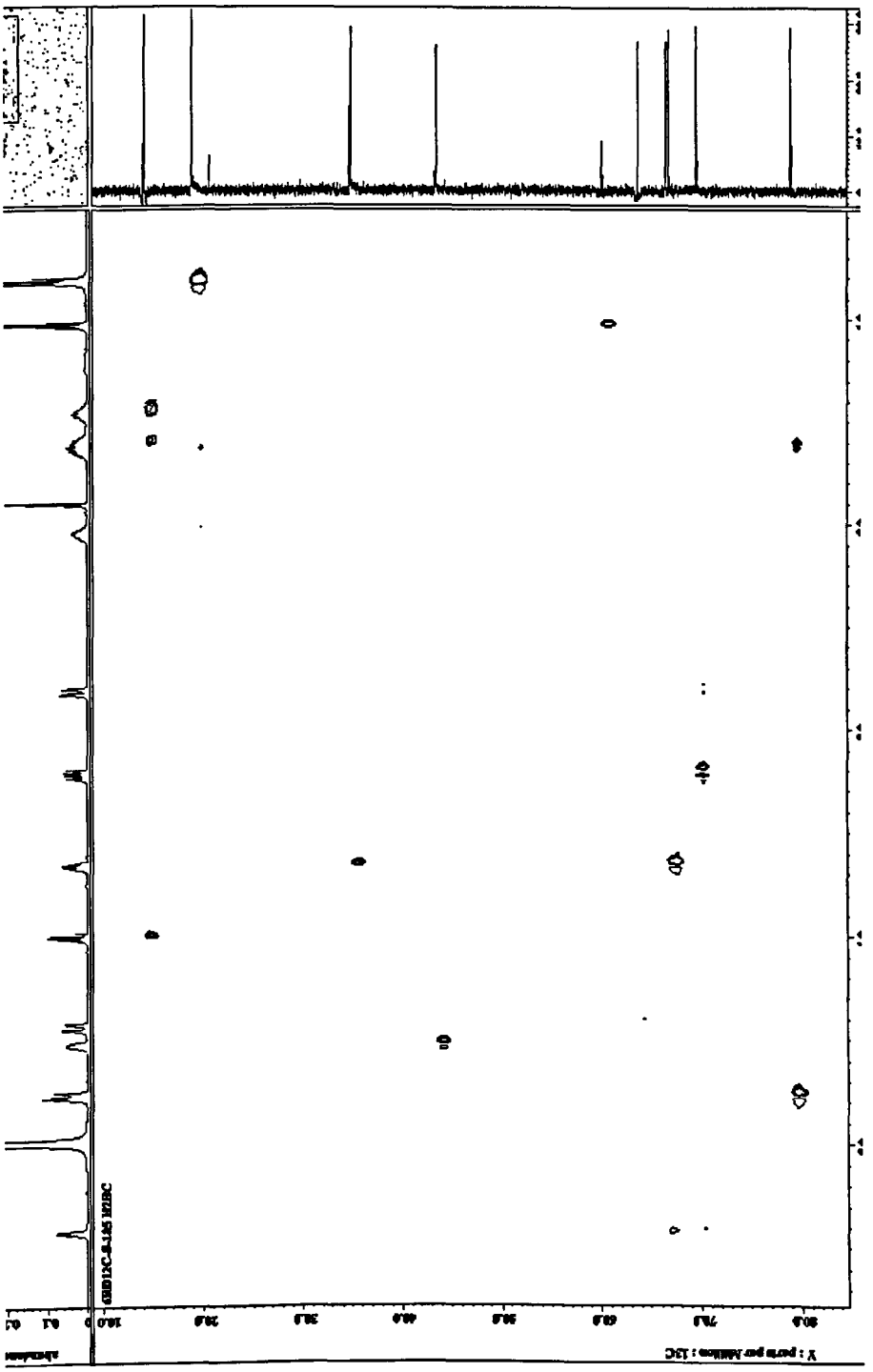


Figure 42 H2BC NMR spectrum of 3.1

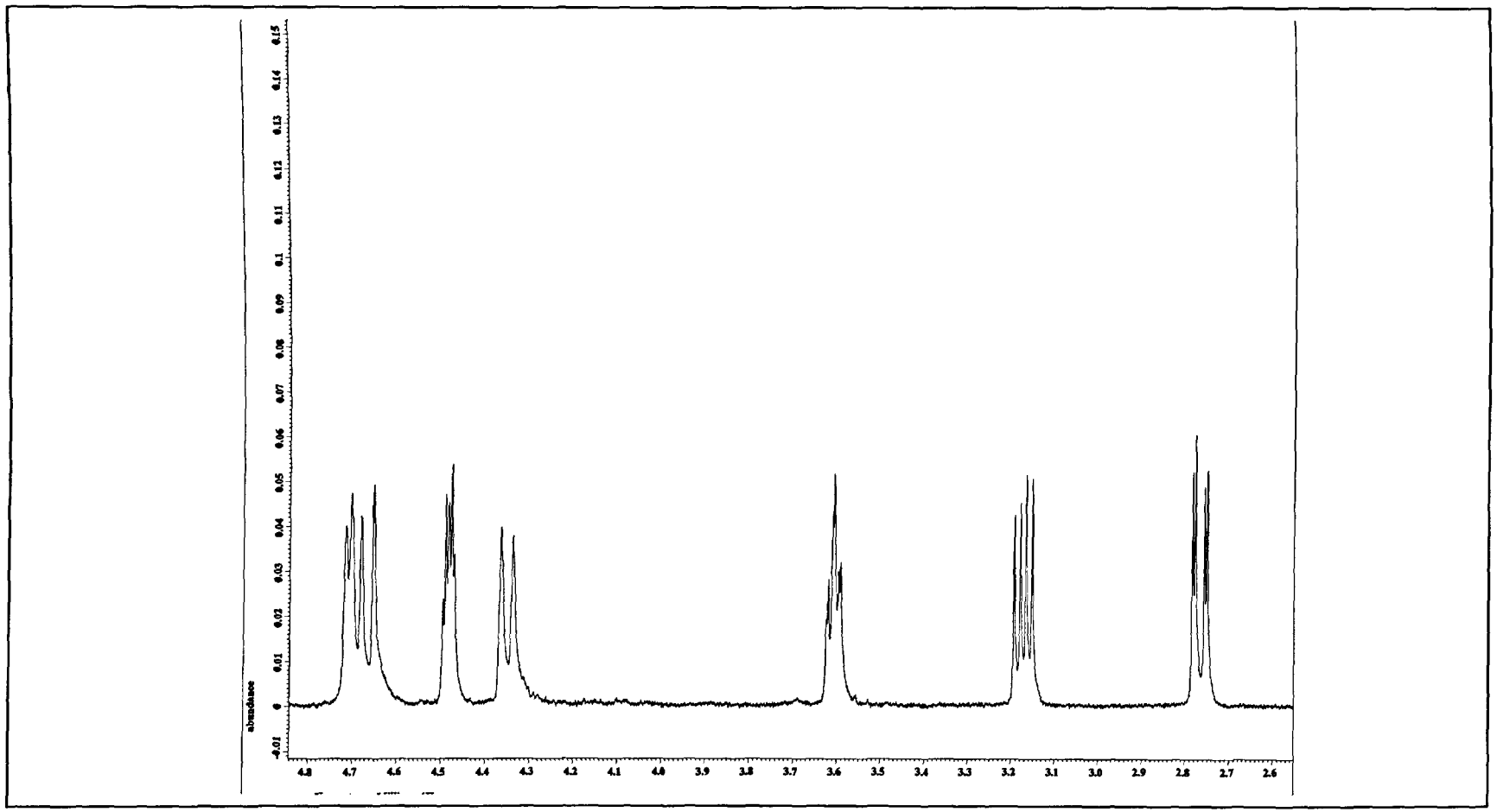


Figure 43 Zoomed ^{1}H NMR spectrum (C_5D_5N + drop of D_2O) of the region at δ_H (2.8 – 4.8) of comound 3.1

3.2 Isolation and structure elucidation of cycloepoxydon 3.2

The second compound (**3.2**) collected from the column described is section 3.1 was a white solid (17.4 mg). Recrystallisation from nitromethane to gave colourless crystals (7.3 mg), mp 186-189 °C, ES $[M+H]^+$ *m/z* 241; $[\alpha]_D^{22}$ - 139° (c 0.25, $CHCl_3$:MeOH (95:5)); $IR_{(ATR)}$ ν_{max} cm^{-1} : 3378, 1673 and. The main IR absorptions suggested the presence of hydroxyl and carbonyl groups.

The ^1H NMR spectrum [$(CD_3)_2CO$, Figure 48] of this compound showed 14 protons at δ_H 0.89 (3H, t, *J* 7.2 Hz), 1.36 (1H, m), 1.38 (1H, m), 1.41(1H, m), 1.79 (1H, m,), 3.19 (td, *J* 8.4, 2.6, Hz), 3.34 (1H, d, *J* 4.1 Hz), 3.85 (1H, t, *J* 3.95), 4.12 (1H, t, *J* 1.96 Hz), 4.15 (2H, m,), and 4.95 (1H, bs).

The ^{13}C and DEPT-135 NMR spectra [$(CD_3)_2CO$, Figure 49] of compound **3.2** revealed a 12 carbon skeleton structure containing: one methyl at δ_C 13.52, three methylene at δ_C 18.49, 34.40, 63.29, five methine carbons δ_C 53.21, 55.12, 63.63, 66.76, 78.27 and three quaternary carbons at δ_C 128.49, 151.79 and 192.73. The ^{13}C NMR and the mass spectral data suggest that this compound is similar in structure to compound **3.1** with a molecular formula of $C_{12}H_{16}O_5$. The main NMR differences in the ^{13}C NMR spectrum of compound **3.1** and **3.2** (Table 10) is the replacement of a methylene signal at δ_C 43.50 in **3.1** by a methine signal at δ_C 54.08 in **3.2**. The other main NMR difference is an up field shift of a methine signal to δ_C 56.55 in **3.2,** whilst all the methine signals resonate at $\delta_C > 65$ in compound **3.1**. The molecular formula $C_{12}H_{16}O_5$ indicates five degrees of unsaturation, which means one more degree of unsaturation compared to compound **3.1**. This information suggested the structure of compound **3.2** to be as shown in Figure 44

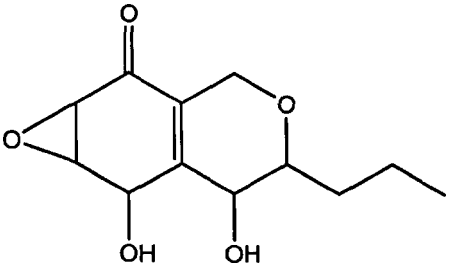


Figure 44 Suggested structure of 3.2

Table 10 ^{13}C NMR data of 3.1 and 3.2 run in pyridine-d5 (C5D5N)

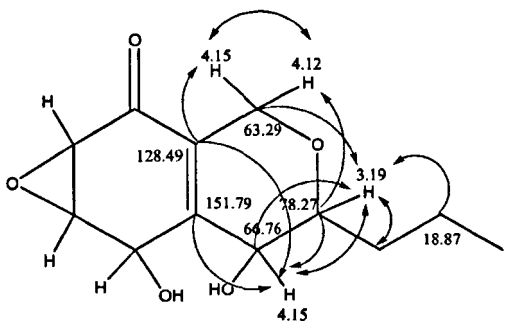
	3.1 NMR DATA	3.2 NMR DATA
No	δ_C	δ_C
1	63.76 – CH_2	63.74 – CH_2
3	79.08 – CH	78.27 – CH
4	66.82 – CH	66.68 – CH
5	66.48 – CH	63.81 – CH
6	69.59 – CH	56.55– CH
7	43.50 – CH_2	54.08 – CH
8	197.08 – C	193.32 – C
9	132.60 – C	129.06 – C
10	155.27 – C	154.02 – C
11	34.91 – CH_2	34.75 – CH_2
12	18.98 – CH_2	18.87– CH_2
13	14.17 – CH_3	14.11– CH_3

A series of 2D NMR experiments were run in acetone-d_6 to confirm the structure. The proposed structure was supported by the HMBC experiment (Figure 52), which showed correlations between the proton at δ_H 3.19 and the carbon at δ_C 18.87. The low field chemical shift of δ_C 78.27 indicates the electronegative effect of the

neighbouring oxygen. Furthermore, an observation of ^{1}H-^{1}H coupling (Figure 51) between the two protons at δ_H 1.38 and 4.15 with that at δ_H 3.19 confirms the neighbourhood of these atoms and the placement of each one.

Further HMBC correlations were observed between δ_C 78.27 and δ_H 4.12, which has a cross peak with δ_C 63.29 in the HMQC spectrum (Figure 50). This carbon in turn showed an HMBC correlation with the methine proton at δ_H 3.19.

The observation of more HMBC correlations between the methylene protons at δ_H 4.15 and 4.12, which are connected to δ_C 63.29 and the carbon signals at δ_C 132.60 and 151.79 allowed the placement of the double bond within the structure as shown by Figure 45

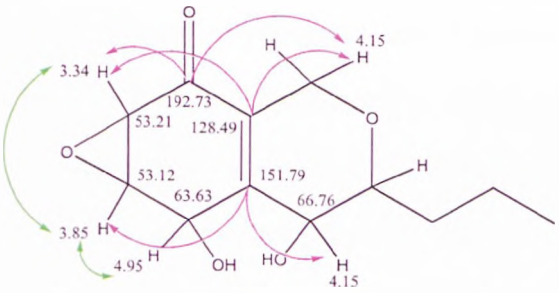


Green arrows: ^{1}H-^{1}H COSY
Pink arrows: HMBC

Figure 45 Partial HMBC and ^{1}H-^{1}H COSY correlation of 3.2

By tracking the rest of the spin system in the ^{1}H-^{1}H COSY and HMBC spectra the whole structure was confirmed. In the ^{1}H-^{1}H COSY the methine proton at δ_H 3.34 (d, *J* 4.1), which showed a cross peak with δ_C 53.21 in the HMQC spectrum, showed coupling with δ_H 3.85 (t, *J* 4.0), which in turn coupled to δ_H 4.95 (bs) as shown in Figure 46. While in the HMBC spectrum, the protons at δ_H 3.85 and 4.15 showed correlation with the carbon at δ_C 151.79. The proton at δ_H 3.34 showed correlations

with the carbon at δ_C 128.49 and 192.73. All these correlations are presented in Figure 46 and Table 11



Green arrows: ^{1}H-^{1}H COSY
Pink arrows: HMBC

Figure 46 The rest of HMBC and ^{1}H-^{1}H COSY correlation of 3.2

Table 11 1D and 2D NMR data for 3.2 run in acetone $(CD_3)_2CO$

No	δ_C	δ_H	HMBC	H-H COSY
1	63.29- CH_2	4.12 (dt, *J* 16.32, 1.96), 4.15(m)	34.40, 192.73, 78.27	4.15
3	78.27- CH	3.19 (td *J* 2.6, 8.4)	66.76, 63.29, 34.40, 18.49	1.38
4	66.76- CH	4.15 (m)	151.79, 128.49, 34.40	3.19
5	63.63- CH	4.95 (bs)	151.79	4.15, 3.85, :
6	55.12- CH	3.85 (t, *J* 3.95)	151.79, 63,63	3.34, 4.95
7	53.21- CH	3.34 (d, *J* 4.1)	192.73, 128.49	3.85
8	192.73- C			
9	128.49- C		3.34	
10	151.79- C			
11	34.40- CH_2	1.38 (m), 1.79 (m)		1.41
12	18.49- CH_2	1.36 (m), 1.41 (m)		0.89
13	13.52- CH_3	0.89 (3H, t, *J* 7.2)		

A literature review revealed, that this compound was isolated for the first time in 1997 by Gehrt *et al* from fermentations of a *Deuteromycete* strain in addition to 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene and 1-hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene [1]. It was given the name cycloepoxydon. The relative stereochemistry of (-)-cycloepoxydon was determined by the NOESY correlations, and by analysis of the ^{1}H-^{1}H coupling constants. By comparing the reported ^{1}H-^{1}H coupling constants with that observed for the isolated compound, it was confirmed that both have the same relative stereochemistry, which means that the isolated compound has the structure shown by Figure 47.

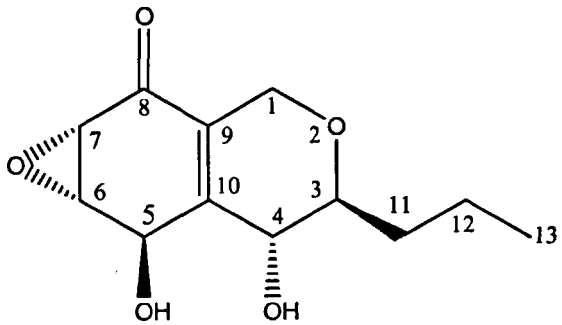


Figure 47 The relative stereochemistry of (-)-cycloepoxydon

Interestingly cycloepoxydon has the same relative stereochemical relationship for the ring substituents as was reported in the previous section for 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one (**3.1**). This suggests that cylcoepoxydon may be the parent compound of compound **3.1**, or *vice-versa.*

Cycloepoxydon was found to be a bioactive inhibitor of eukaryotic signal transduction and can be a candidate for antitumor drugs, especially against breast cancer [2-3]. The total synthesis of this compound was also reported [4-5].

So this is the second report for the isolation of cycloepoxydon from a natural source, and the first report for the isolation of this compound from an endophytic xylaria fungus. This fungus was re-cultured twice to test the stability of its metabolite production. In both cases the fungus produced the same metabolites. The mycelium was examined for its content, and it was found to contain the same metabolites as well as the culture medium.

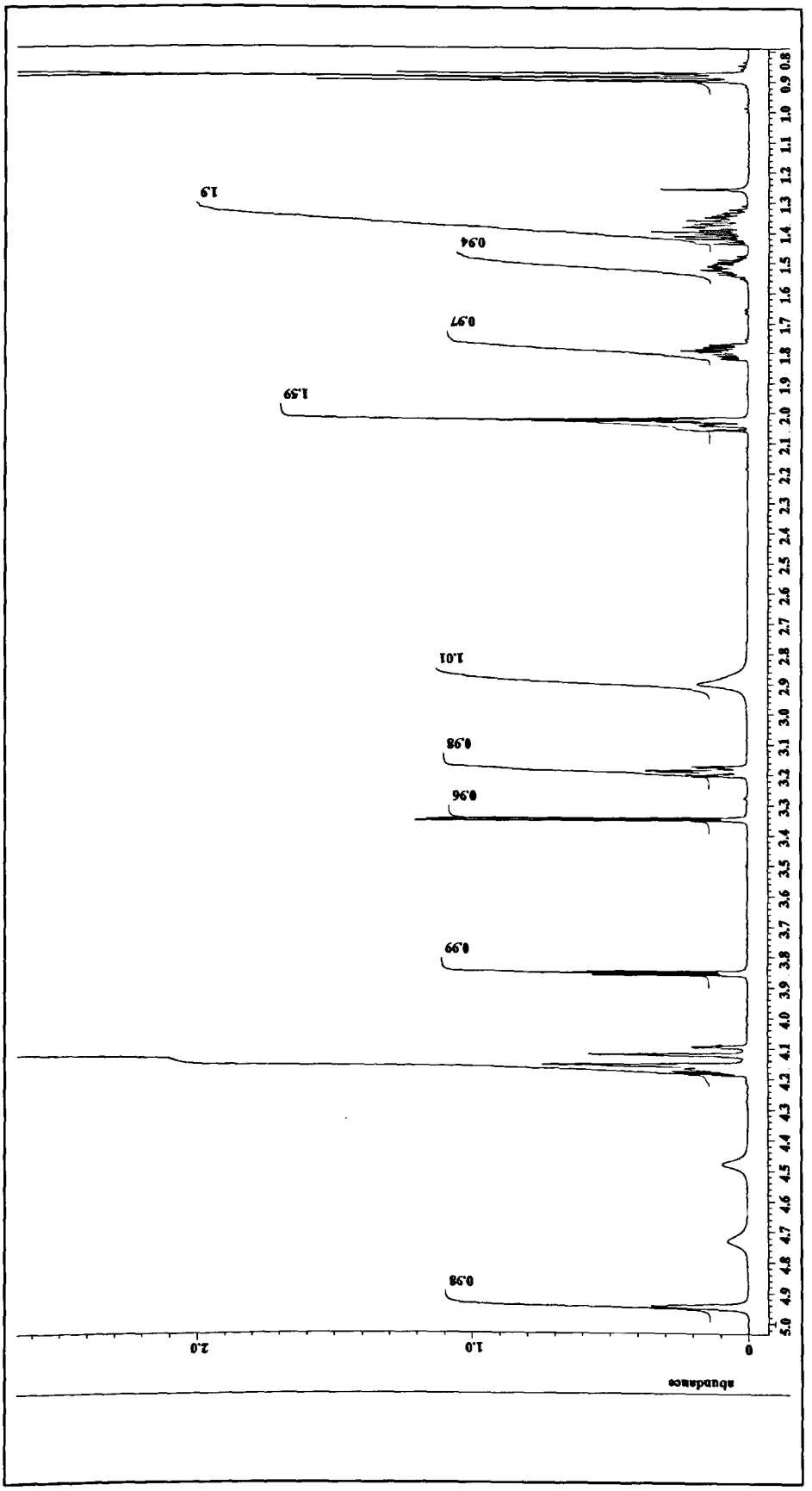


Figure 48 ^{1}H NMR spectrum $(CD_3)_2CO$ of 3.2

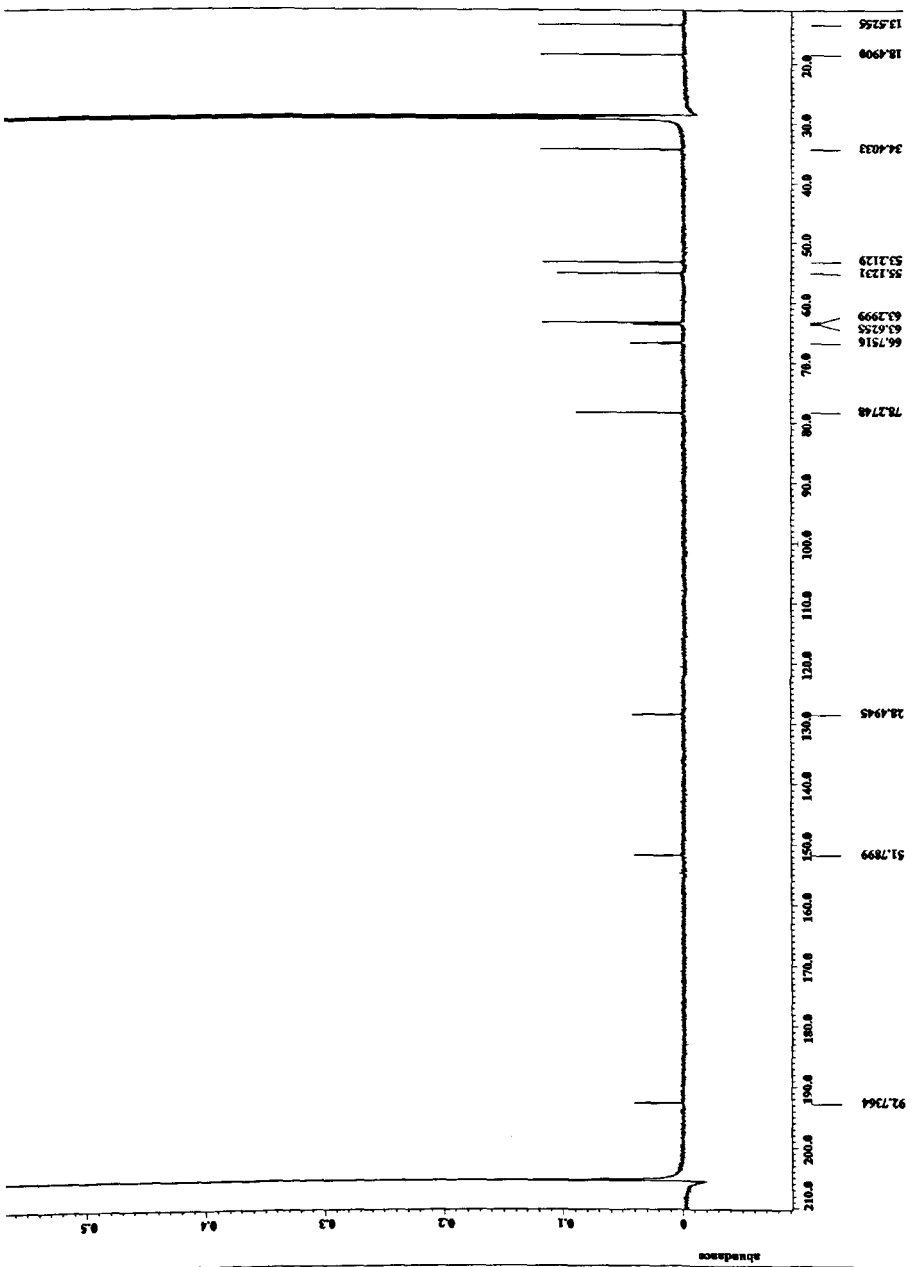


Figure 49 ^{13}C NMR spectrum $(CD_3)_2CO$ of 3.2

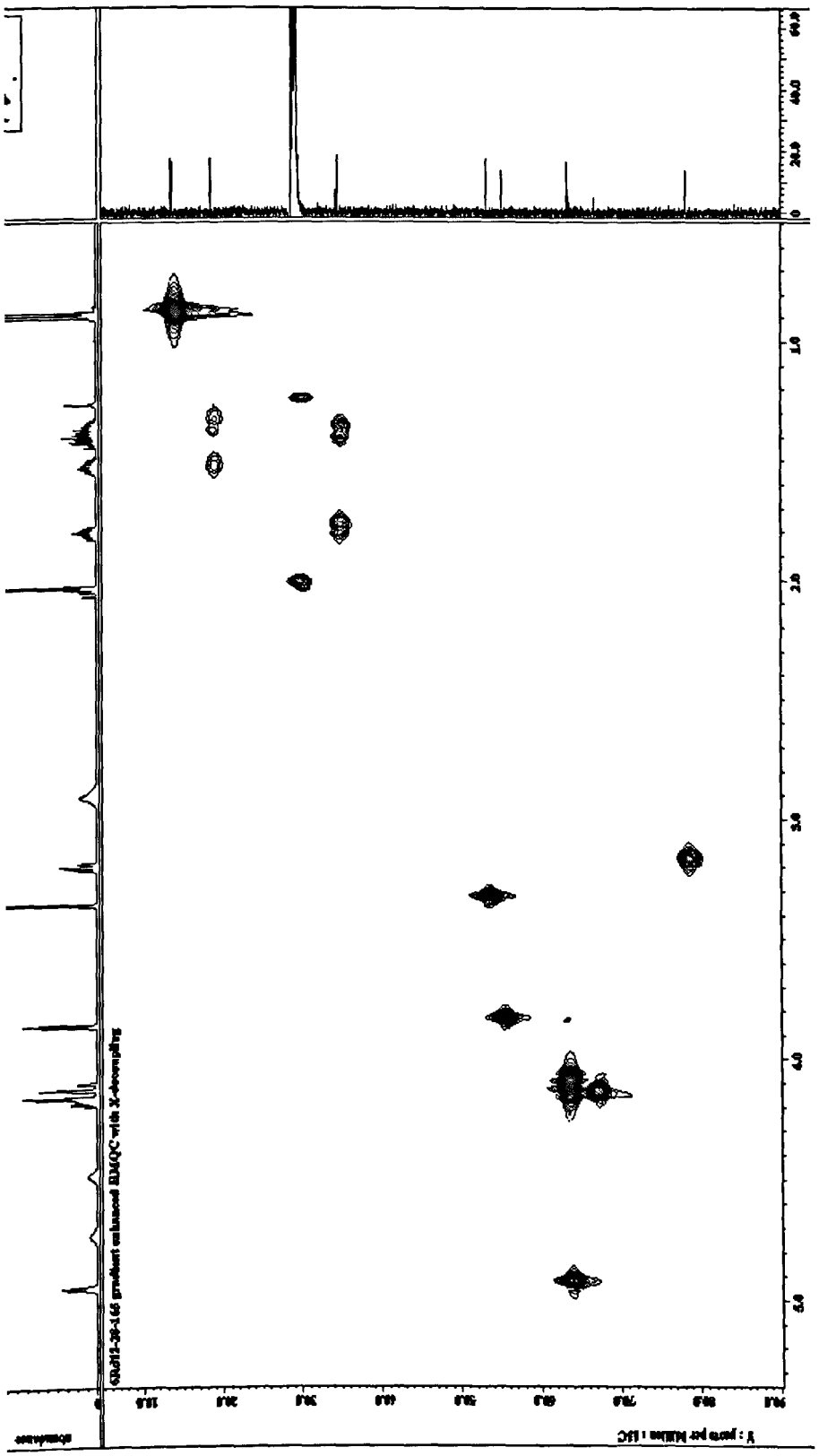


Figure 50 HMQC NMR spectrum for 3.2

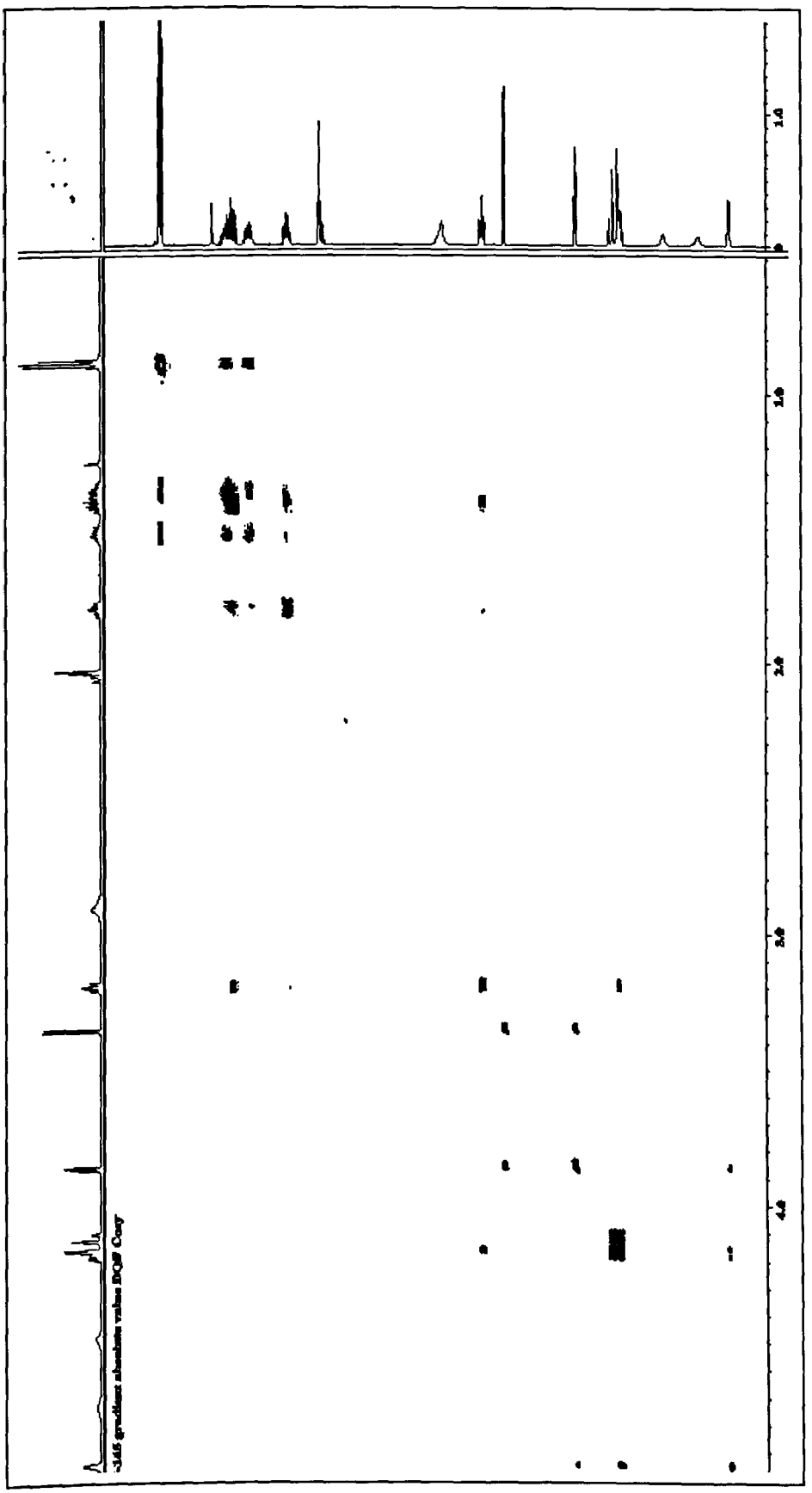


Figure 51 ^{1}H-^{1}H COSY spectrum for 3.2

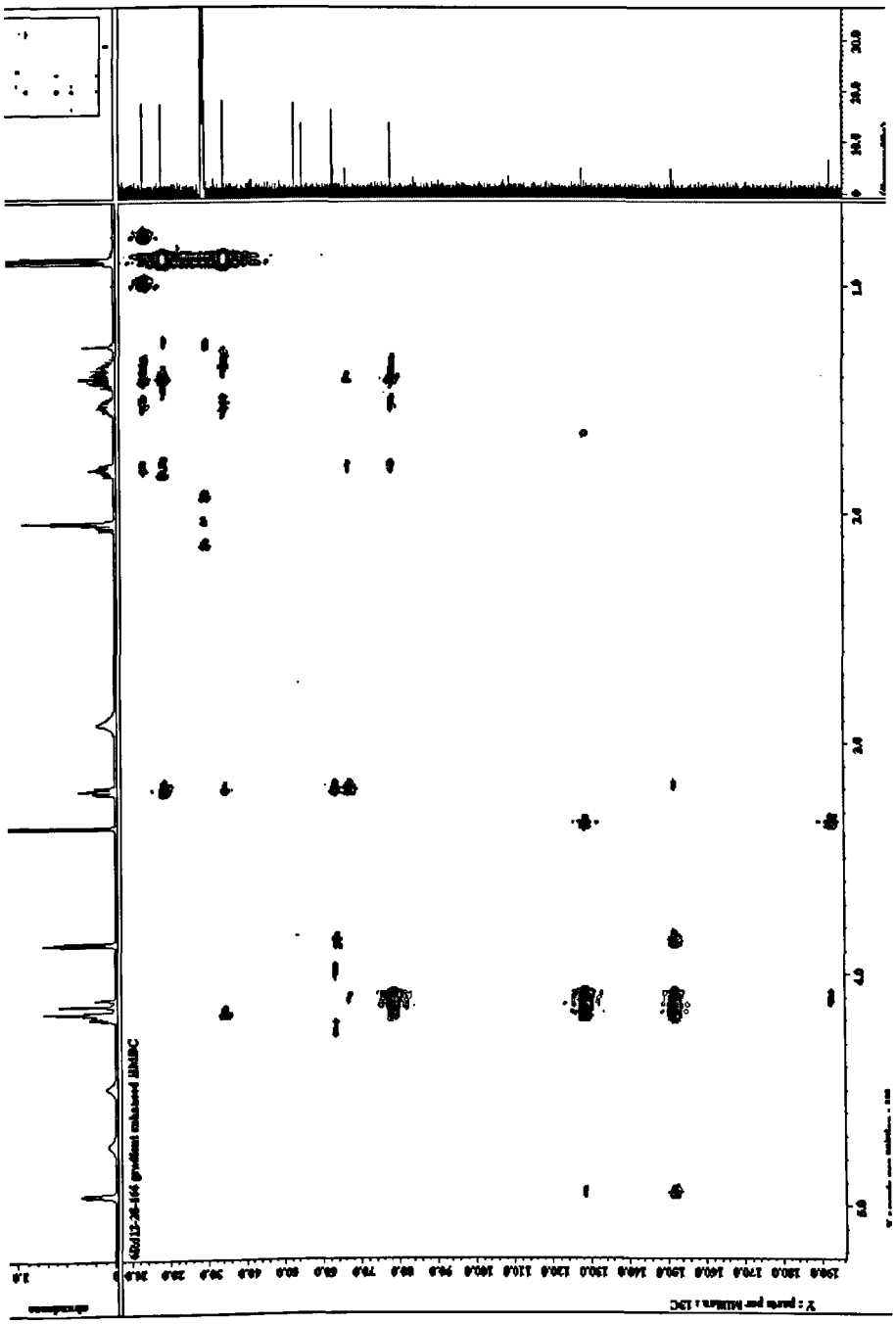


Figure 52 HMBC spectrum of 3.2

References

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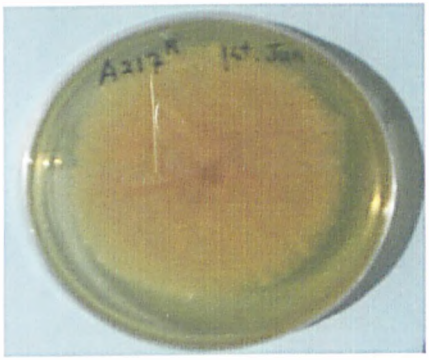
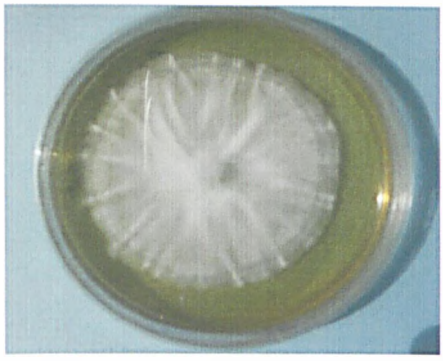
Chapter 4 Secondary metabolites from X. A217R and A517R

4.0 Fungi A217R and A517R overview

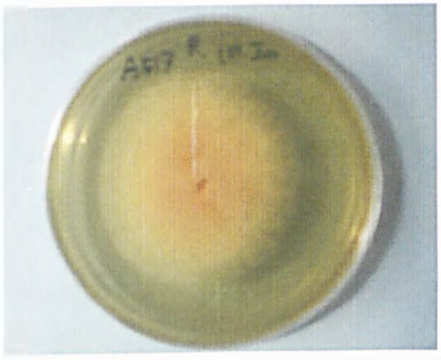
The fungi coded A217R and A517R are endophytes extracted from palm trees (*Arenga pinnata* (Sugar Palm)) from Kao Loung National Park in Thailand. They were received in Petri dishes. They looked similar to each other from back and front views of the Petri dishes as shown in Figure 53.



A217R back view



A217R front view



A517R back view

A517R A217R front view

Figure 53 A217 R and A517 R in Petri dishes.

Both fungi were sub-cultured in conical flasks containing aqueous 3% malt extract to prepare them for large scale culturing. After 3 weeks the conical flask containing

A217R was showing stromata with a black base and a white tip, whilst A517R showed flower-like stamen stromata as shown in Figure 54.

Each fungus was cultured in six Thomson bottles (2L) in aqueous 3% malt extract enriched with 6% glucose for eight weeks; each bottle containing 1 litre of culture medium.

A217R conical flask A517R conical flask

Figure 54 Fungi A217R and A517R in conical flasks after 3 weeks

The mature fungi formed white gelatine-like myceliums (Figure 55). The mycelium was removed by filtration through a muslin cloth and the aqueous filtrate extracted with ethyl acetate (3 x 700 ml) in a 5 L separating funnel. The extract was dried with sodium sulphate for 30 min. and the ethyl acetate was removed on a rotary evaporator to give a brownish gummy material (A217R 5.5 g and A517R 6.2 g).

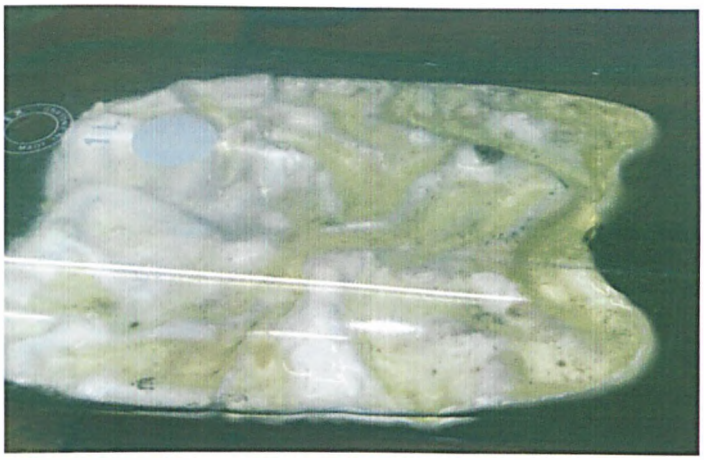


Figure 55 Mycelium of Fungus A517R

The extracts were then analyzed for their content by TLC. A solvent system of toluene: ethyl acetate: acetic acid (50:49:1) was used as eluent. Both extracts showed matching patterns of spots under visible and UV light. Under visible light, a violet coloured spot appeared at R_f of 0.58. Under short wave UV (245 nm), three UV active spots were detected; a weaker spot at the lower R_f 0.27 and two stronger spots at 0.65 and 0.90.

When the TLC plates were sprayed with *p*-nitroaniline the two upper UV active bands (R_f 0.65 and 0.90) developed a strong orange colour spots and an opaque spot at R_f 0.27.

Comparison of the spots patterns of both A217R and A517R, suggested that both extracts were from very similar fungi species that produce similar secondary metabolites.

4.1 Isolation of Cytochalasin D from fungus A217R

The ethyl acetate extract was triturated with ethyl acetate and left overnight. A white precipitate was obtained. The white solid compound was filtered off and

recrystallized from ethyl acetate to give white needles (65 mg), mp 238-240 °C, ES $[M+H]^+$ *m/z* 508. This compound showed an opaque spot when treated with diazotised *p*-nitroaniline spray reagent at R_f 0.27 on the TLC. This compound was also isolated by preparative TLC run with a solvent system of toluene: ethyl acetate: acetic acid (50:49:1) by recovery of the band at R_f 0.27.

The IR spectrum (Figure 56) of this compound shows the following distinguishable signals: 3681, 3405, 2919, 1739 and 1689 cm^{-1}, which can indicate the presence of the followings: an amide group, a carbonyl ester group, and a hydroxyl.

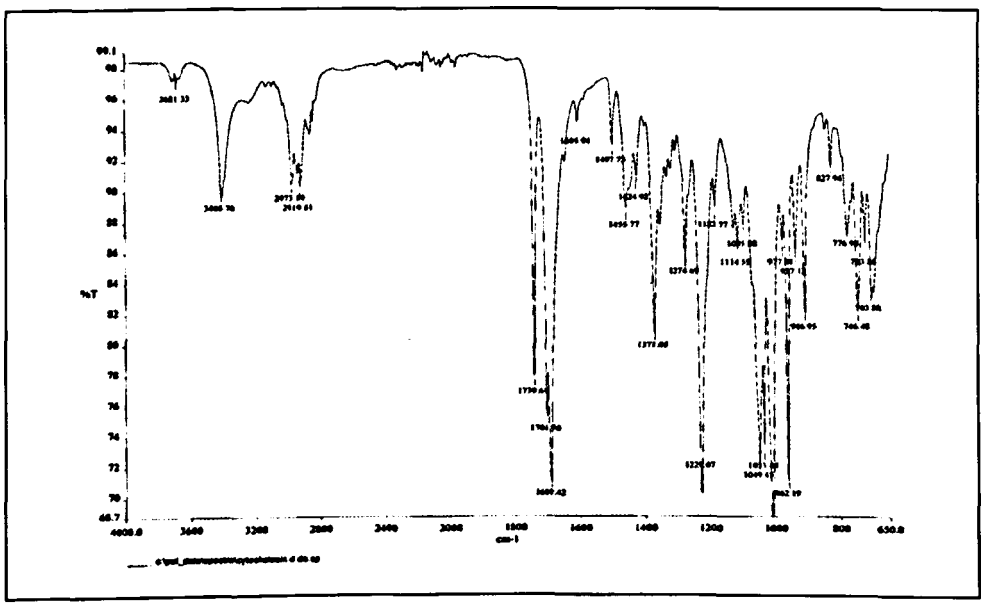


Figure 56 IR spectrum of cytochalasin D.

^1H NMR spectrum (C_5D_5N) of this compound (Figure 58) gave 4 methyl protons at δ_H 0.77 (d, *J* 6.70 Hz), 0.98 (d, *J* 6.50 Hz), 1.50 (s) and 2.33 (s). it also revealed 3 methylene protons at δ_H 1.93 (dd, *J* 5.0, 11.8 Hz), 2.86 (m), 5.41 (s) and methyne protons at δ_H 2.42 (m), 2.66 (m), 2.97 (m) 3.38 (t, *J* 10.5 Hz), 3.55 (m), 4.39 (d,

J=10.1 Hz), 5.60 (m), 5.57 (m), 6.01 (d, *J* 15.5), 6.34 (m), 6.84 (dd, *J* 2.23, 15.8 Hz), 7.14 (m) and 7.24 (m).

The protons at δ_H 7 region indicated the presence of a phenyl ring, whilst the shifts between δ_H 5.5 and 6.5 indicated possible alkene protons with adjacent protonated carbons, based on the splitting patterns. The singlet peaks at δ_H 5.05 and 5.41 did not show any correlations in the ^1H-^1H COSY spectrum except with each other; they were therefore recognised as peaks from a terminal alkene carbon linked to a non-protonated carbon.

The ^{13}C (Figure 59), DEPT-135 and DEPT-90 NMR spectrum run in (C_5D_5N) of this compound presents 28 carbon signals. The signals at δ_C 128.79 and 129.90 are double the height of other peaks signifying that these signals are each due to two carbon atoms, to give a total of 30 carbons. There are four methyl signals at δ_C 13.59, 19.34, 24.61 and 20.51, three methylene signals at δ_C 45.49, 112.17 and 38.54, seven quaternary signals at δ_C 210.88, 175.11, 170.47, 151.63, 138.44, 78.39, and 53.99, plus 17 signals due to methyne carbons; seven methine signals in the olefinic/aromatic region between δ_c 126-134 and nine further methine carbon signals in the region of δ_c 13-80.

The spectral and physical data of this compound suggested this compound was a cytochalasin, which are commonly formed as secondary metabolites by Xylariaceous fungi. The comparison of this ^1H and ^{13}C NMR data with the reported NMR data for a series of cytochalasins revealed that this compound is cytochalasin D ($C_{30}H_{37}NO_6$, Figure 57). The proton signal observed at δ_H 9.10 and the methylene signal in the DEPT 135 spectrum at δ_C 112.17, which showed a cross peak to δ_H 5.05 and 5.41 in the HMQC, are indicative of cytochalasin D (Figure 57).

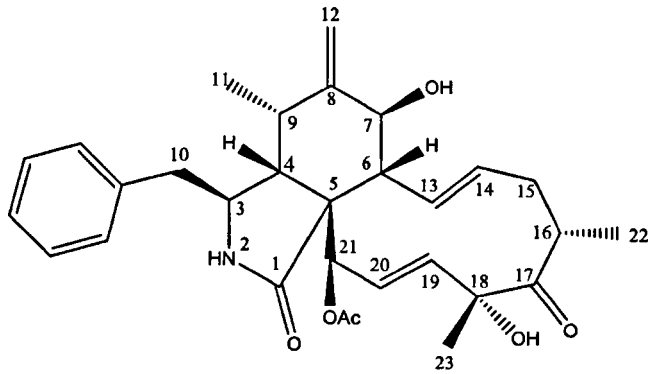


Figure 57 Cytochalasin D

The chemical shift of the N-H proton of the lactam ring in cytochalasin compounds appears generally above δ_H 9.0, when pyridine-d_5 is used as the NMR solvent. It has been observed that the signal for this proton can be characteristic for cytochalasin (Table 12). In cytochalasin D for instance, the N-H proton resonates at δ_H 9.10, which is the case for the isolated cytochalasin.

Table 12 Characteristic physical data of some cytochalasins [1].

Cytochalasin	Amide δ_H in pyridine	mp °C	*m/z*
C	9.38	260- 265	507
19,20-epoxycytochalasin C	9.46	245-247	523
D	9.13	267-270	507
N	9.55	272	523
O	9.25	258- 265	525
P	9.10	169-173	525
Q	9.45	145-147	507
R	9.7	159-167	523

1D and 2D NMR experiments were used for the C-H assignments. Data from ^1H NMR, ^{13}C, DEPT, and HMQC of this cytochalasin are presented in (Table 13).

Cytochalasin D was reported for the first time as a metabolite of *Metarrhizium anisopliae* [2]. Its structure determination using chemical methods was reported in 1969 [3]. From that time it is known as a common secondary metabolite produced by fungi. It possesses antibiotic [4] and antitumor [5] activities. It also weakens maintenance of long term potentiation (LTP) of actin filaments [6].

Table 13 ^{1}H NMR, ^{13}C, DEPT, and HMQC (C_5D_5N) of cytochalasin D

NO	δ_C	DEPT	δ_H (mult)
1.	175.11	C	
2.	NH		9.10
3.	54.36	CH	3.55 m
4.	50.07	CH	2.42 m
5.	33.14	CH	2.97 m
6.	151.63	C	
7.	71.27	CH	4.39 d (*J* 10.1)
8.	47.87	CH	3.38 t (*J* 10.5)
9.	53.99	C	
10.	45.49	CH_2	2.86 m
11.	13.59	CH_3	0.77 d (*J* 6.70)
12.	112.17	CH_2	5.05, 5.41 s
13.	126.79	CH	5.60 m
14.	132.80	CH	5.57 m
15.	38.54	CH_2	1.93 dd (*J* 5.0, 11.8)
16.	42.43	CH	2.66 m
17.	210.88	C	
18.	78.39	C	
19.	133.82	CH	6.34 d
20.	132.95	CH	6.84 dd (*J* 2.23, 15.8)
21.	77.99	CH	6.01 d (*J* 15.5)
22.	19.34	CH_3	0.98 d (*J* 6.5)
23.	24.61	CH_3	1.50 s
24.	170.47	C	
25.	20.51	CH_3	2.33 s
26.	138.44	C	
27.	129.90	CH	7.24 m
28.	128.79	CH	7.14 m
29.	127.84	CH	7.22 m
30.	128.79	CH	7.14 m
31.	129.90	CH	7.24 m

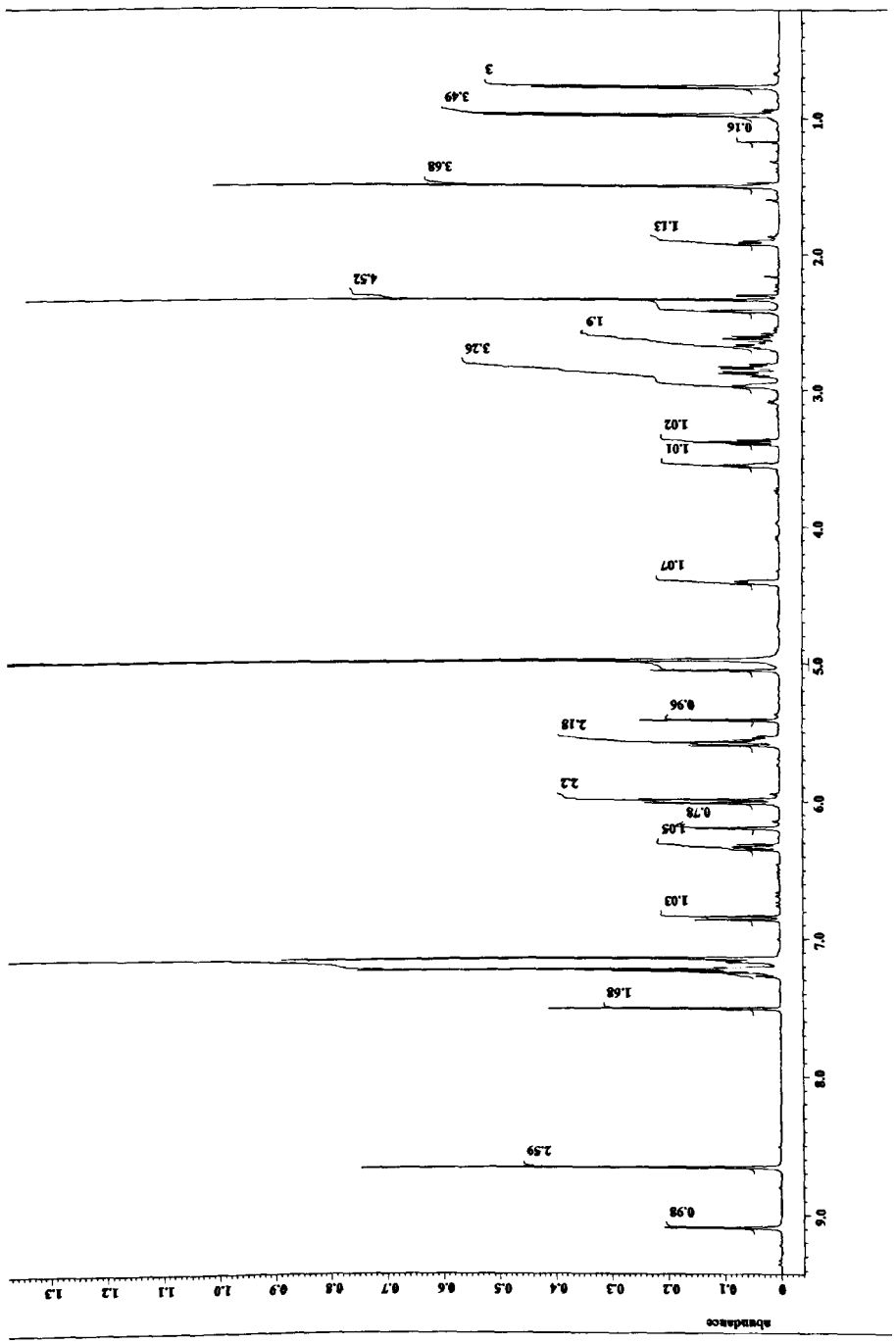


Figure 58 ^{1}H NMR spectrum of cytochalasin D in C_5D_5N

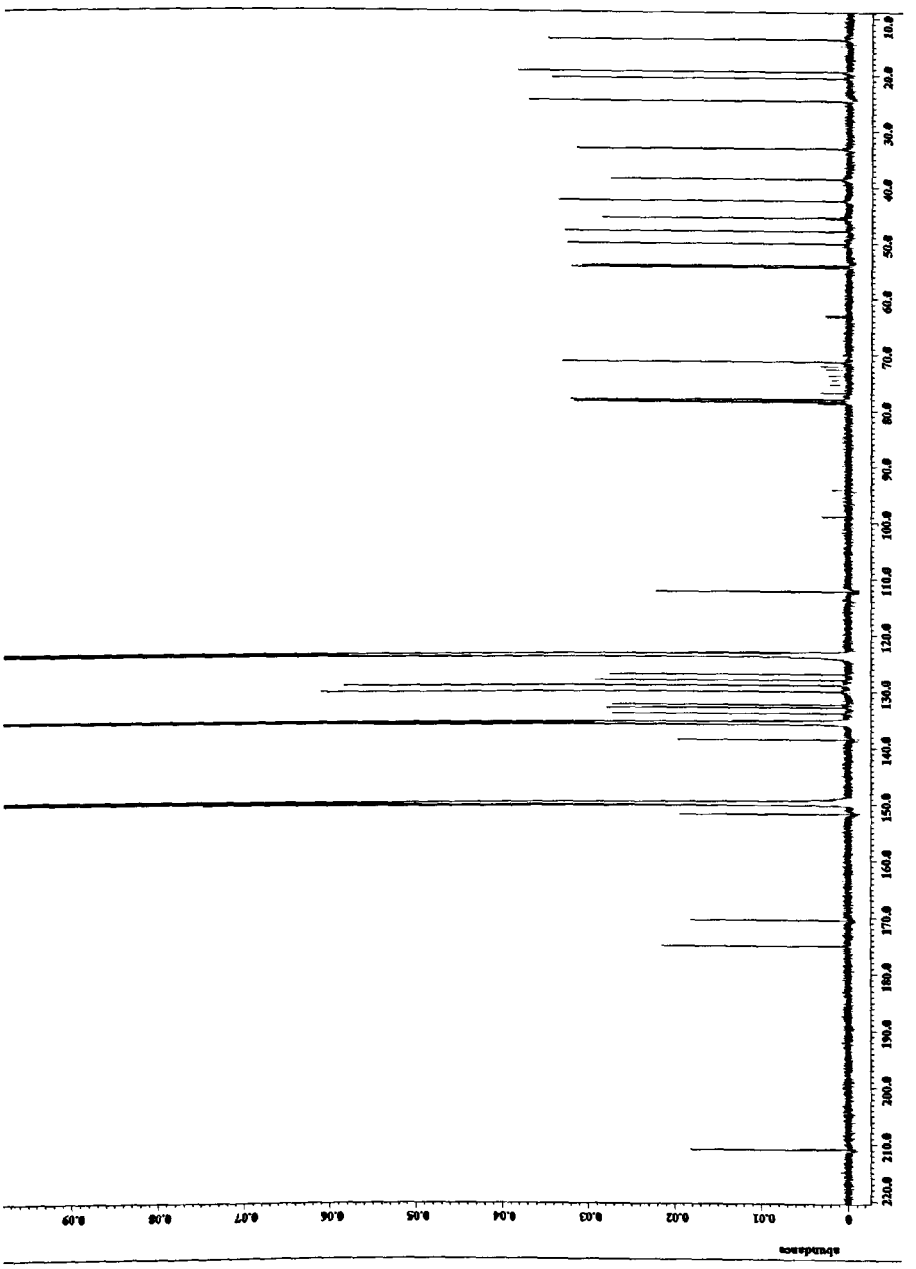


Figure 59 ^{13}C NMR spectrum of cytochalasin D in C_5D_5N

4.2 Isolation of Mellein from fungus A217R

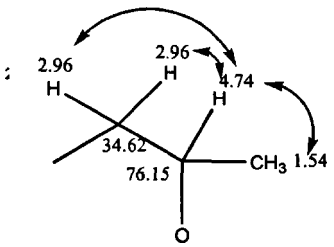
After the isolation of cytochalasin D, the remaining ethyl acetate crude extract obtained from the fungus A217R (4.0 g) was triturated with warmed toluene to give a yellow oily fraction (1.1 g). This fraction showed 2 main compounds at at R_f 0.9 and 0.65 The resultant yellow oily extract was subjected to preparative TLC (8 plates 20×20 cm, GF254 silica gel), eluted with toluene: ethyl acetate: acetic acid (50:49:1).

The UV active band at R_f 0.90 developed a strong orange colour, when sprayed with diazotised *p*-nitroaniline spray reagent suggesting a phenolic compound. The band was recovered and washed with ethyl acetate to produce yellow solution. Removing the solvent gave pale yellow oil (34 mg). Trituration of the yellow oil with *n*-hexane gave a white solid (7.5 mg), which was recrystallised from the same solvent to give white plates (4.0 mg), mp 53-55 °C, ES $[M+H]^+$ *m/z* 179, $[\alpha]_D^{23}$ + 85° (c 0.05, MeOH); $IR_{(ATR)}$ ν_{max} cm^{-1} 3187, 2997, 1662, 1614 and 1198. The IR absorptions suggested the presence of a hydroxyl and carbonyl groups.

The ^1H NMR ($CDCl_3$, Figure 62) shows ten proton signals at δ_H 1.54 (3H, d, *J* 6.27 Hz), 2.96 (2H, d, *J* 7.13 Hz), 4.74 (1H, m, *J* 8.09, 6.42), 6.72 (1H, d, *J* 7.39, Hz), 6.91 (1H, d, *J* 8.51 Hz), 7.44 (1H, dd, *J* 7.39 and 8.42 Hz), 11.03 (1H, s).

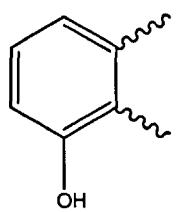
In the ^{13}C NMR spectrum ($CDCl_3$, Figure 63) ten carbon signals comprise a methyl carbon at δ_C 20.80, a methylene at 34.62, four methine carbons at 76.15, 116.23, 117.95, and 136.19, and four quaternaries at 108.29, 139.34, 162.17, and 170.0. From the mass spectrum, ^1H and ^{13}C NMR, the molecular formula was deduced to be $C_{10}H_{10}O_3$.

In the ^1H-^1H COSY spectrum, the doublet methyl proton at δ_H 1.54, which correlates to δ_C 20.80 in the HMQC spectrum, showed coupling to a methine proton at δ_H 4.74, which correlates to δ_C 76.15 in the HMQC. The down field carbon signal at δ_C 76.15 suggested an adjacent oxygen atom. The methine proton at δ_H 4.74 also coupled to the methylene protons at δ_H 2.96 which showed a cross peak with δ_C 34.62 in the HMQC spectrum. This gives a partial structure X.



X

The three protons in the aromatic region at, 7.44, 6.91 and 6.72 suggested a phenolic ring supported by the orange spot given by the compound, when sprayed with diazotised *p*-nitroaniline spray reagent in the TLC. Also six aromatic carbons were detected at δ_C 108.29, 116.23, 117.95, 136.19, 139.43 and 162.17, three of which are quaternary carbons according to the DEPT135 analysis; suggesting a 1,2,3-trisubstitued benzene ring; partial structure Y.



Y

The singlet proton at δ_H 10.96 is a characteristic signal of a chelated –OH group. The carbon signal at δ_C 170.00 suggested an ester carbonyl carbon. According to the low

infra-red absorption at 1662 cm^{-1} a chelated lactone system was likely. Combining the two substructures gave the structure of mellein (Figure 60).

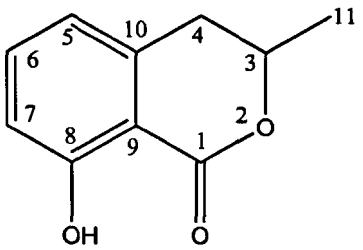


Figure 60 Structure of mellein

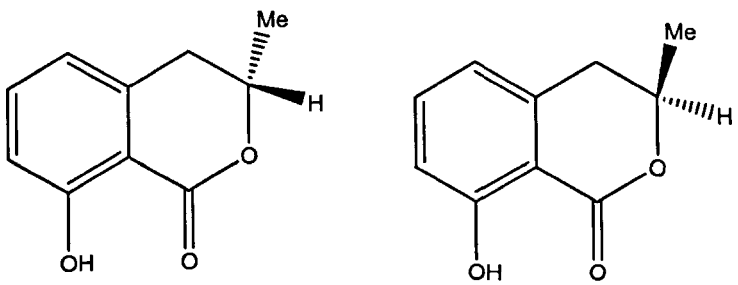
The NMR data of the compound were established unambiguously using 1D and 2D NMR spectral experiments, ^{1}H, ^{13}C, ^{1}H-^{1}H COSY and HMQC experiments (Table 14).

Table 14 The ^{1}H and ^{13}C NMR assignments of mellein.

No	δ_C	δ_H
1	170 0 – C	
3	76.15 – CH	4.74 (m)
4	34.62 – CH_2	2.96 (d, *J*= 7.13 Hz)
5	117.95 – CH	6.91 (d, *J*= 8.51 Hz)
6	136.19 – CH	7.44 (dd, J= 8.42, 7.39 Hz)
7	116.23 – CH	6.72 (d, *J*= 7.39)
8	162.17 – C	
9	108.29 – C	
10	139.43 – C	
11	20.80 – CH_3	1.54 (d, *J* 6.20 Hz)
*	Chelated OH	11.05 (s, OH)

The first isolation of this compound was reported in 1933 from Aspergillus *melleus* [7-8]. It has also been isolated in Bradford from different Xylaria fungi. such as X. *Longiana* [9], X. *grammica* [10] and X. *badia* [11]. In this project mellein has been found to be produced by several xylaria fungi species, which means that mellein is a common secondary metabolite from xylaria fungi. The yield of mellein from most of the fungi is very low compared to other metabolites. It has also been found that fungi which produce this metabolite can produce other derivatives such as 4-hydroxymellein and 8-*O*-methylmellein.

Dimitriadis *et al* reported [12] the stereospecific synthesis to both enantiomers of mellein, where the optical rotation of the (S)-(+)-mellein is $[\alpha]_D^{25}$ +88.6 (c 0.27, MeOH) and for (R)-(-)-mellein is $[\alpha]_D^{25}$ - 101.3 (c .07, $CHCl_3$) (Figure 61). Islam *et al* reported the synthesis of (R)-(-)-mellein with the optical rotation as $[\alpha]_D^{21}$ - 102 (c 0.53, $CHCl_3$) [13]. By comparing the above optical rotation with that of the isolated mellein, the latter was confirmed as (S)-(+)-mellein.



S-Mellein R-Mellein

Figure 61 S and R enantiomers of mellein

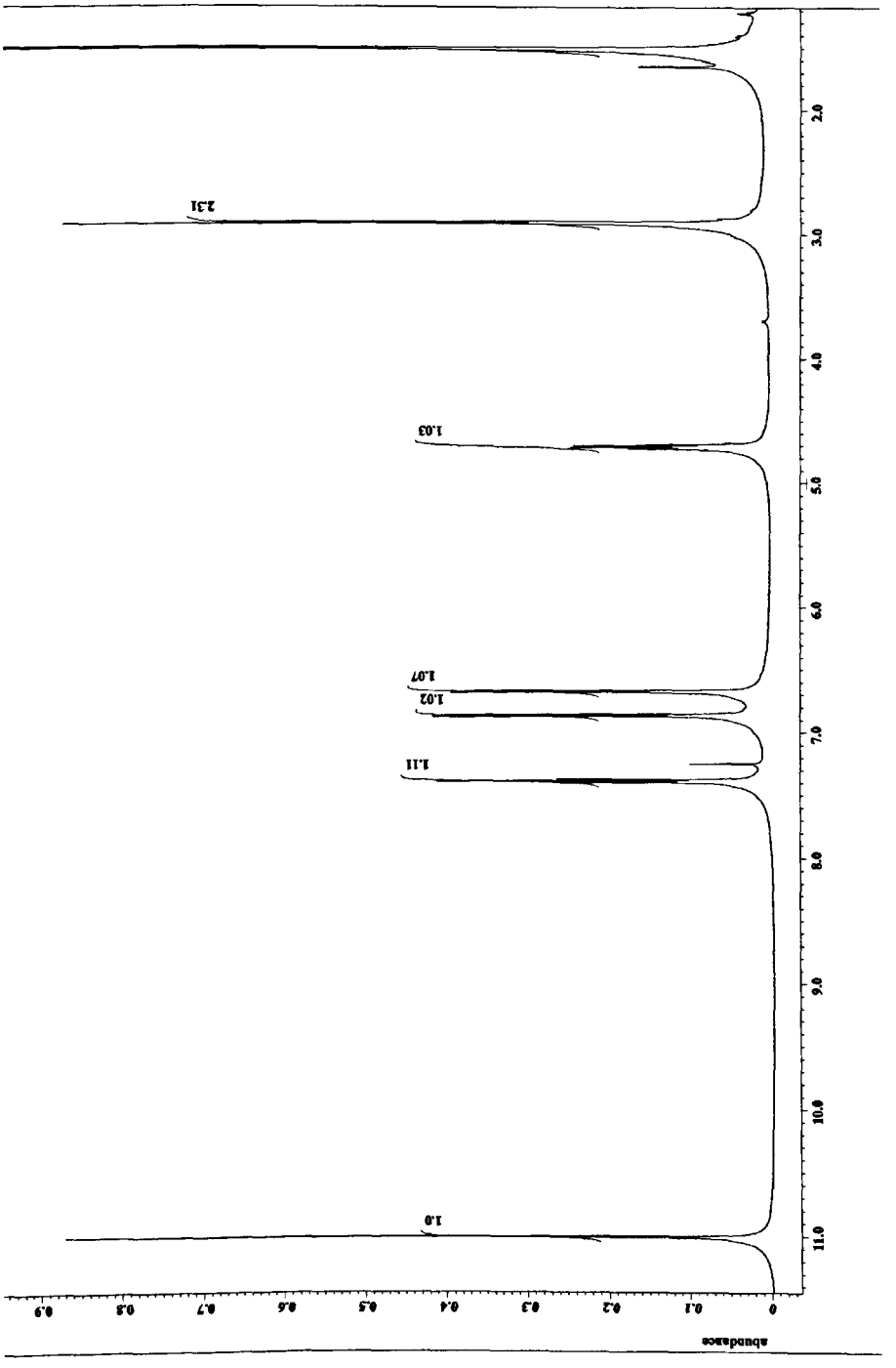


Figure 62 ^{1}H NMR spectrum of mellein in $CDCl_3$

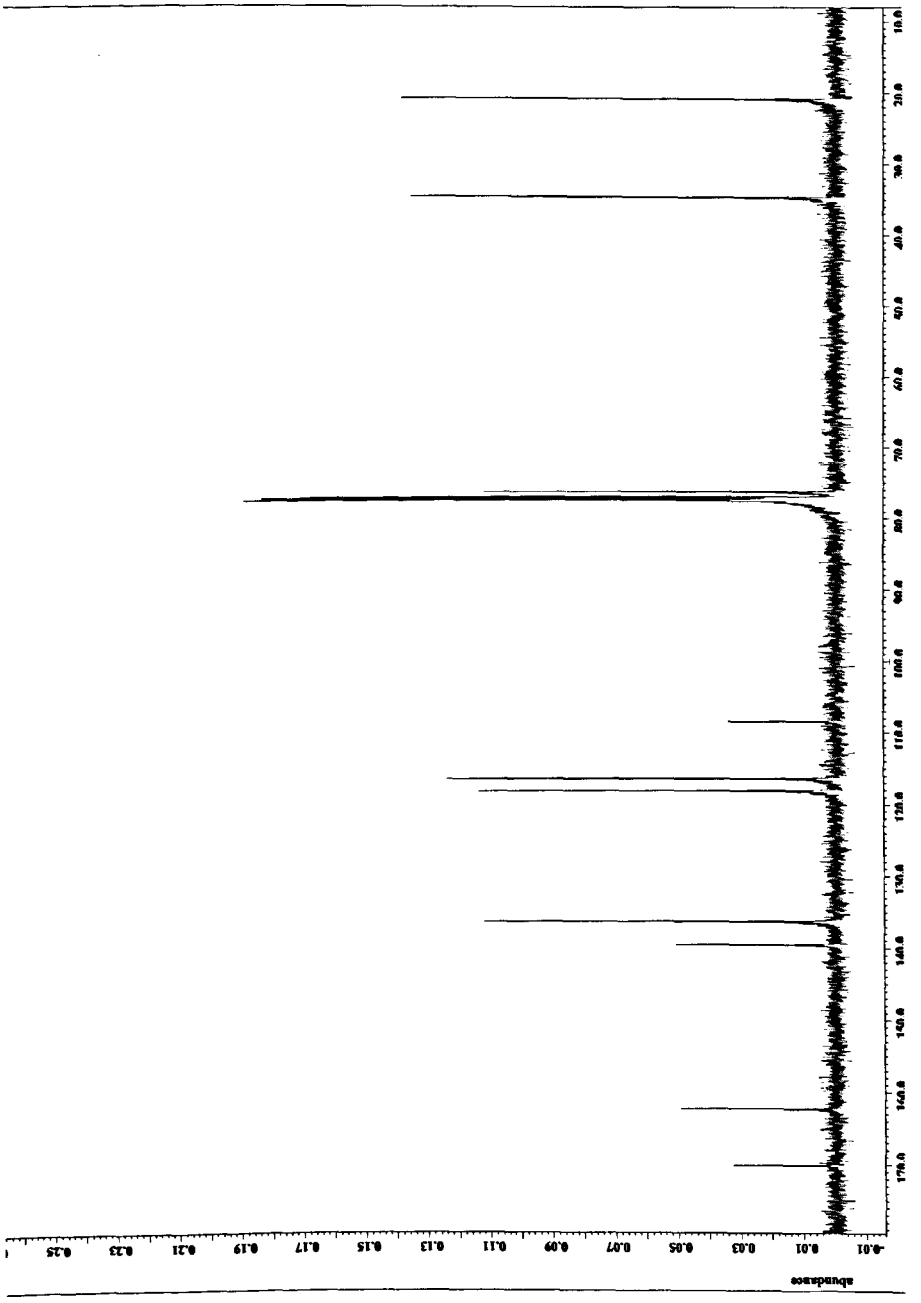


Figure 63 ^{13}C NMR spectrum of mellein in $CDCl_3$

4.3 Isolation of *cis*-4-Hydroxymellein from fungus A217R

The next UV active band in the PTLC at R_f 0.64, which also developed a strong orange colour when sprayed with diazotised *p*-nitroaniline spraying reagent; suggesting a phenolic compound, was recovered. The recovered silica was washed with ethyl acetate to give a yellow solution. The ethyl acetate was removed under low pressure to produce yellowish needles (255 mg), which were recrystallized from toluene to give white needles, 119 mg; mp. 117-120 °C; m/z 195 (M^+); $[\alpha]_D^{23}$ + 38 (c 0.5, MeOH); IR: ν_{max} cm^{-1} 3427, 3178, 2997, 1675. IR absorptions at 3427 and 3178 cm^{-1} in the spectrum of this compound indicated the presence of two hydroxyl groups. The latter suggested a hydrogen bonded hydroxyl group, whilst the absorption at 3427 cm^{-1} indicated a non-hydrogen bonded OH.

In the ^1H NMR in $CDCl_3$ (Figure 66) ten protons were indicated at δ_H 1.58 (3H, d, *J* 6.6 Hz), 2.18 (s (br)), 4.55 (1H, d, *J* 2.1 Hz), 4.68 (1 H, qd, *J* 2.1 Hz, *J* 6.62 Hz), 6.91 (1H, d, *J* 7.30 Hz), 7.51 (1H, dd, *J* 8.51 and 7.30 Hz), 7.01 (1H, dd, *J* 8.51 Hz), 10.97 (1H, s).

The ^{13}C NMR ($CDCl_3$, Figure 67) revealed ten carbons, which comprise a methyl carbon at δ_C 16.15, five methine at 67.37, 78.33, 118.44, 118.67, and 136.94, and four quaternaries at 106.93, 140.60, 162.20, and 169.33.

From the mass spectrum, ^1H and ^{13}C NMR, the molecular formula was deduced to be $C_{10}H_{10}O_4$, where there were two hydroxyl groups, one of which occurs at δ_H 2.18 and another at δ_H 10.97. The latter indicates a chelated hydroxyl group. Because of the yellow spot of this compound with diazotised *p*-nitroaniline, a hydroxyl group on the aromatic ring was likely. This information together suggested a mellein type compound. By comparing *m/z* of mellein to that of this compound, it is 16 unit larger

than that of mellein, indicating an additional oxygen. The absence of a methylene signal in the carbon spectrum of this compound compared to that of mellein indicates this compound is substituted at C-4. The replacement of the methylene carbon in mellein with a down field methine carbon at δC 78.33 suggested the substitution at C-4 to be a hydroxyl group, which means that this compound is 4-hydroxymellein. Comparison of the ^{1}H and ^{13}C NMR data of this compound with literature data showed it to be *cis*-4-hydroxymellien [14-16] (Figure 64).

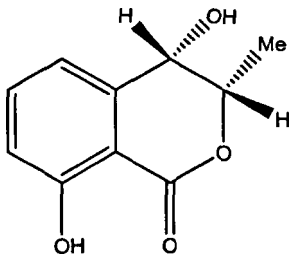


Figure 64 cis-4-hydroxymellien

The *cis* or *trans* configuration of the protons at C-3 and C-4 in 4-hydroxymellein is based on the coupling constant between the two protons. A coupling constant of 2 Hz is consistent with the *cis* form, whereas a 4 Hz coupling constant is associated with the *trans* form.

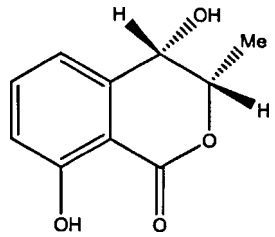
The 3-H, 4-H coupling constant was observed to be 2.10 Hz. On this basis, the *cis*-configuration was assigned. A spin decoupling NMR experiment was performed by irradiation of the methyl group protons at position C-11. This eliminated the coupling between the methyl group protons and the proton at position C-3. The quartet of doublet signal at 4.68 Hz was "collapsed" leaving a doublet peak. The *J*-coupling between the 3-H and 4-H measured at J= 2.08 Hz confirmed the coupling behaviour detected in the ^{1}H NMR spectrum.

The NMR data of the compound were established unambiguously using 1D and 2D NMR spectral experiments, ^{1}H, ^{13}C, COSY-45 and HMQC experiments. HMQC revealed all H-C direct correlations and thus confirmed the assignment of all carbons with attached protons (Table 15).

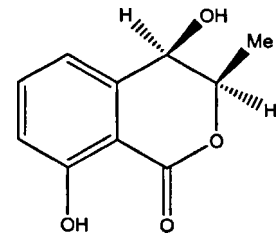
Table 15 The ^{1}H and ^{13}C NMR assignments for 4-Hydroxymellein.

No	δ_C	δ_H
1	169.33 – C	–
3	67.37 – CH	4.68 (qd, *J* 6.62, 2.10)
4	78.33 – CH	4.55 (d, *J* 2.10 Hz)
5	118.67 – CH	6.91 (d, *J* 7.30)
6	136.94 – CH	7.51 (dd, *J* 8.51, 7.30)
7	118.44 – CH	7.01 (d, *J* 8.51)
8	162.20 – C-OH	10.97 (s, -OH)
9	106.93	–
10	140.60	–
11	16.15 – CH_3	1.58 (d, *J* 6.6)

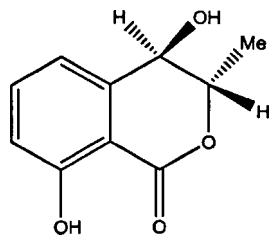
Because of two chiral centers in the molecule, four enantiomers are possible (Figure 65).



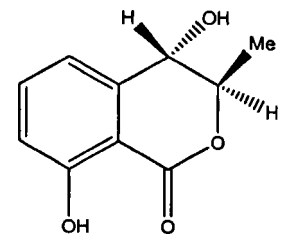
(3S,4S)-4-hydroxcymelein (**A**)



(3R,4R)-4-hydroxcymelein (**B**)



(3S,4R)-4-hydroxcymelein (**C**)



(3R,4S)-4-hydroxcymelein (**D**)

Figure 65 4-hydroxymellein enantiomers

4-Hydroxymellein of undetermined stereochemistry has been isolated from *Aspergillus oniki* [17], *Anisospora camptospora* [18], and *Aspergillus ochraceus* [19].

cis-3*S*,4*S*-4-hydroxymellein (**A**) in addition to mellein have been isolated from the mycelium of *Cercospora taiwanensis*, grown on potato-agar. [20].

3R,4R-4-Hydroxymellein (**B**) was found in *Lasiodiplodia theobromae* [18], *Septoria nodorum* [21], *Aspergillus melleus* [22-23], *Botryosphaeria obtuse* [24] and from the mycelium of the coprophilous fungus *Apiospwa monkzgnei* [25]

It also has been isolated from *Papulaspora immersa*, which was found in roots and leaves of *Smallanthus sonchifolius*. It showed strong synergistic cytotoxic activity with (22E,24R)-8,14-epoxyergosta-4,22-diene-3,6-dione against the human tumor cell lines MDA-MB435 (melanoma), HCT-8 (colon), SF295 (glioblastoma), and HL-60 (promyelocytic leukemia) [26],

3S,4R-4-Hydroxymellein (**C**) was isolated in Bradford University from the fungus *Xylaria grammica* [27]. 3R,4S-4-Hydroxymellein (**D**) has been isolated from the

medicinal plant *Moringa oleifera* [28] and together with the 3R,4R isomer from the fungus *Septoria* [29].

This isomer was also isolated from the marinederived fungus *Cladosporium sp* [30].

Table 16 summarizes the optical rotation of each enantiomer. This identifies the isolated compound in this report as 3S,4S-4-Hydroxymellein. This the second report of this isomer.

Table 16 4-Hydroxymellein reported in the literatures

Enantiomer	mp (°C)	$[\alpha]_D^T$ (°)	Source
3S, 4S (**A**)	118-119	+37.4° (c 0.33, MeOH at 20 °C)	*Cercospora taiwanensis* [20]
	117-120	+38° (c 0.5, MeOH at 23 °C)	This case
3R, 4R (**B**)	114-115	-41±3° ($CHCl_3$ at 20 °C)	*Septoria nodorum* [21]
	112-117		*Lasiodiplodia theobromae* [18]
	133.5-135	-27.5° (c 0.04, $CHCl_3$ at 23 °C)	*Aspergillus melleus* [22-23]
	113-114	-39.6° (*c* 0.59, MeOH at 20 °C)	*Microsphaeropsis* strain [31]
3S, 4R (**C**)	135	+17.87° (c 0.71, $CHCl_3$ at 23 °C)	*xylaria grammica* [27]
		+7.08° (c 0.53, MeOH at 23 °C)	
3R, 4S (**D**)	131-132	-28 (c 0.5, MeOH at 23 °C)	*Cladosporium sp* [30]
		-13° (*c* 0.53, $CHCl_3$ at 20 °C)	*Microsphaeropsis* strain [31]

Cis-4-hydroxymellein was tested for bioactivity [32-33]. It showed good activity against the alga *Chlorella fusca,* and against the Gram positive *Bacillus megaterium*. It also showed antifungal activity against *Microbotryum violaceumm,* which suggests that the endophytic fungus could protect the host by producing metabolites, which may be toxic or even lethal to phytopathogens and highlights the potential of endophytic fungi in producing bioactive metabolites [32-33] .

(3*R*,4*R*)-4-hydroxymellein and (3*R*,4*S*)-4-hydroxymellein were both isolated from *Sphaeropsis sapinea* [34]. They showed a synergistic activity, when assayed for phytotoxic and antifungal activities on host and non-host plants [34].

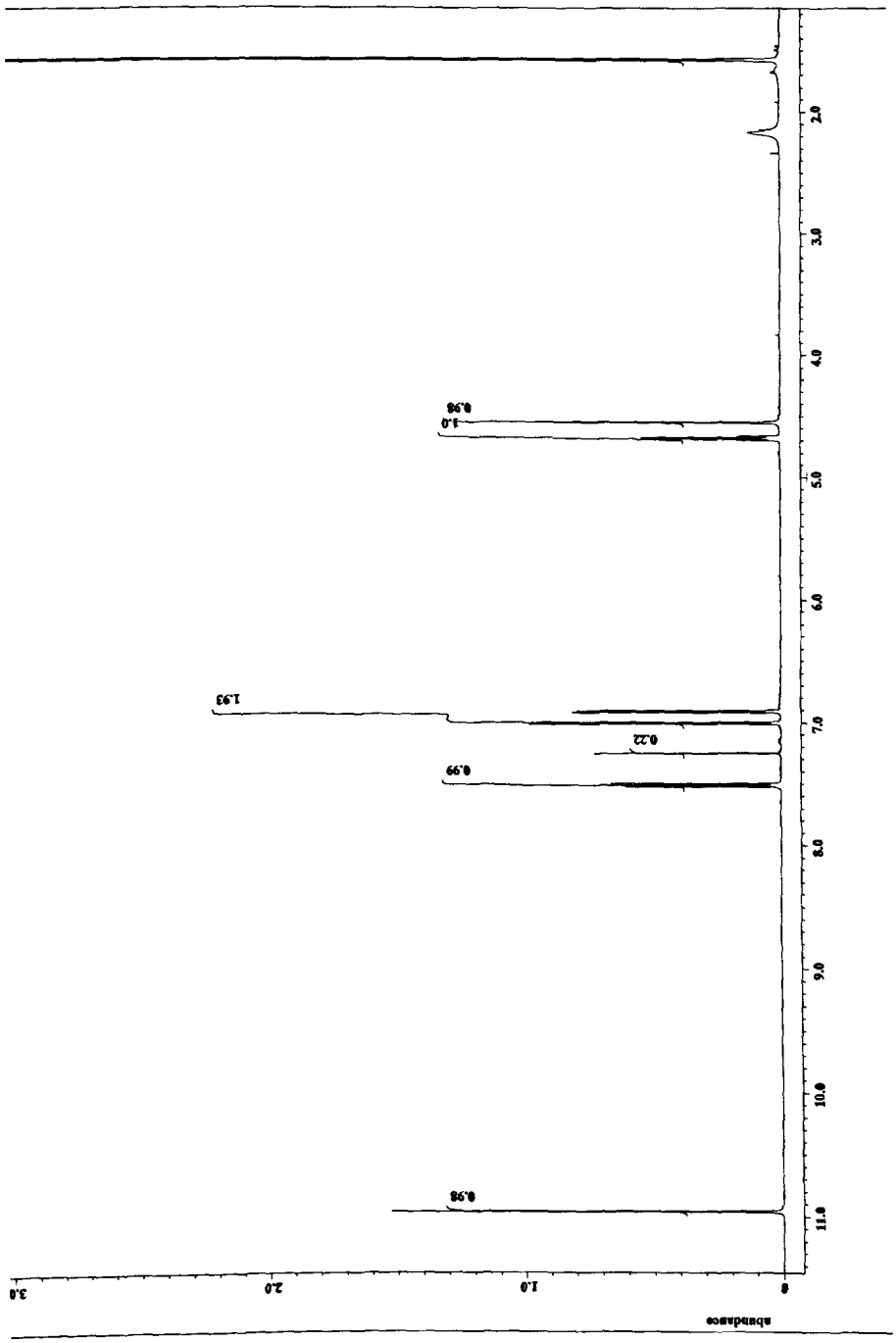


Figure 66 ^{1}H NMR spectrum of cis-4-Hydroxymellein

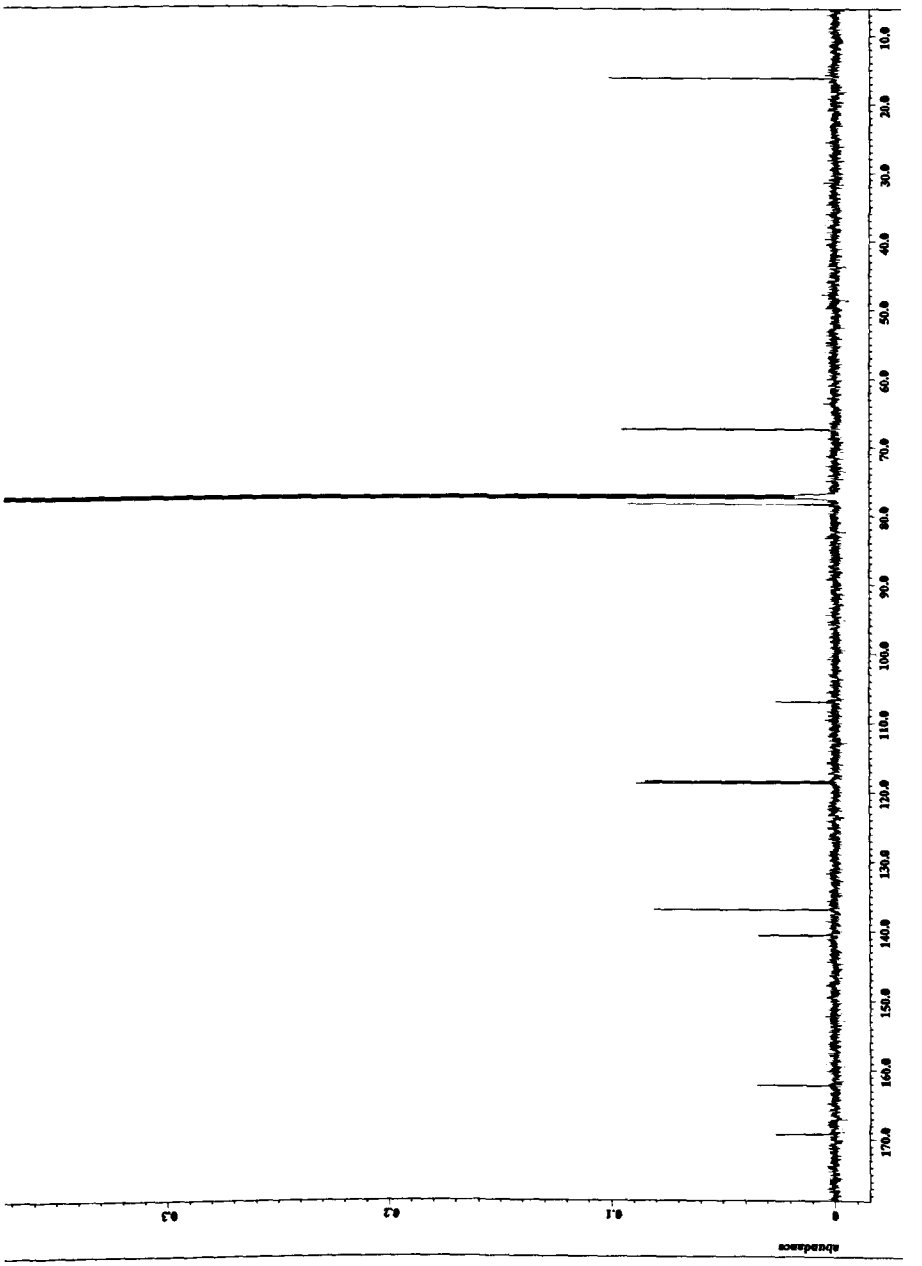


Figure 67 ^{13}C NMR spectrum of cis-4-Hydroxymellein.

4.4 Secondary metabolites from fungus A517R

The same procedures described for the isolation of secondary metabolites of A217R were applied to the ethyl acetate crude extract of the fungus A517R. It was found that this fungus produced exactly the same secondary metabolites as A217R, which means that the two fungi were the same species.

The mycelia of A217R and A517R did not show any significant quantities of secondary metabolites.

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Chapter 5 Secondary metabolites from fungus *X*.X04

5.0 Overview of Xylaria species X04:

Fungus X04 was collected from Thailand and classified as a *Xylaria* species (*Xylaria* cf. *juruensis*). The mycological data provided is summarised in Table 17. Figure 68 shows 3 microscopic photos of X04.

Table 17 Description of X04

Host	Dicot
Location	Trad Province, Thailand
Stromatal size (cm)	1.7-3.0 cm long x 0.2-0.3 cm in diameter
Stromatal shape	Cylindrical, with brown peeling layer, an acute sterile apex, roughened with perithecial contours, stipe with short hair
Texture	Woody
Surface colour (outer)	Brownish black
Surface colour (inner)	White
Perithecial shape	Spherical to obovoid
Ascospore colour	Brown to darkbrown
Ascospore shape	Inequilateral narrowly to broadly rounded ends
Ascospore size	9.0-12.0 x 3.0-4.5 um

Figure 68 Microscopic images of X04

The fungus was cultured for eight weeks on aqueous malt extract solution (3%) (16 L) with added glucose (6%) in 32 × 1 L Thompson bottles. The fungus produced a white mycelium with gelatinous body and cylindrical shaped stromata with a black base as seen in Figure 69

Figure 69 X04 fungus growing in a conical flask

The contents were filtered through a muslin cloth. The mycelium was left to air dry. The aqueous culture medium was extracted in batches with ethyl acetate (3×700 ml) using a 5 L separating funnel. The combined extracts were dried over anhydrous sodium sulphate. Removal of ethyl acetate under reduced pressure using a rotary evaporator gave a dark brown oily gum (8.5 g). The content of the viscous gum was examined by TLC using a number of different solvent systems.

The resultant plates showed that the solvent systems:- toluene : ethyl acetate : acetic acid (50 : 49 : 1), chloroform : methanol (95 : 5) and petroleum ether (60-80 °C) : ethyl acetate : acetic acid (50 : 50 : 1) gave relatively similar separation patterns. The latter system was chosen.

5.1 Isolation and structure elucidation of coriloxin

Dark brown crystals were observed inside the gum from the ethyl acetate extract. The gum was triturated with toluene and gave needle-like crystals, which were

isolated and recrystallised twice from toluene to give colourless needles (2.5 g), mp 153-155 °C; ES $[M+H]^+$ *m/z* 171; $[\alpha]^{23}_D$ -98° (c 0.5 in MeOH); IR_{KBr} ν_{max} cm^{-1} 3398, 2942, 1650, 1606 .

The ^1H NMR ($CDCl_3$) spectrum (Figure 74) showed ten protons at δ_H 5.26 (d, *J* 2.02 Hz, 1H), 4.49 (d *J* 4.55 Hz, 1H), 3.77 (s, 3H), 3.34 (d *J* 2.02 Hz, 1H), 2.59 (d, *J* 4.55 Hz, 1H) and 1.66 (s, 3H). The signal at δ_H 5.26 is indicative of an olefinic methine proton, whilst the signal at δ_H 4.49 indicates a methine proton adjacent to oxygen. The singlet signal at δ_H 3.77 suggested a methoxy group.

The ^{13}C NMR ($CDCl_3$) spectrum (Figure 75) revealed eight carbon signals comprising two methyl signals at δ_C 18.93 and 56.58, three methine signals at δ_C 60.57, 69.12 and 98.20 and three quaternary signals at δ_C 59.44, 171.41 and 193.41. The NMR data together with the ES $[M+H]^+$ *m/z* 173 suggested the presence of 4 oxygen atoms to give a possible molecular formula of $C_8H_{10}O_4$.

The signal at δ_C 193.41 can be assigned to a conjugated carbonyl group, which is supported by an IR absorption at 1650 cm^{-1}. The ^{13}C NMR signals at δ_C 56.58, 59.44, 60.57, and 69.12 suggested carbons adjacent to oxygen atoms. Excluding the carbonyl signal at δ_C 193.41, 3 oxygen atoms are left for 4 carbons, which suggests the presence of epoxide group.

The spectral and physical data of this compound was compared with literature data and identified this compound as coriloxin (Figure 70). The ^1H, ^{13}C, DEPT-135 and HMQC NMR data are listed in Table 18.

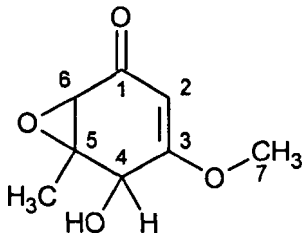


Figure 70 Coriloxin

Table 18 ^{1}H and ^{13}C NMR data of Coriloxin

No	δ_C	Multiplicity	δ_H
1	193.41	C	
2	98.20	CH	5.26 (1H, d, 2.02 Hz)
3	171.41	C	
4	69.12	CH	4.49 (1H, d, 4.55 Hz)
5	59.44	C	
6	60.57	CH	3.34 (1H, d, 2.02 Hz)
7	56.58	CH_3	3.77 (3H, s)
8	18.93	CH_3	1.66 (3H, s)
9	OH	OH	2.69 (d, 4.55 Hz)

Coriloxin was first isolated from Coriolus *Vernicipes* fungi in Japan [1]. It was also reported from unpolished rice fermented with a xylariaceous endophytic fungus [2]. The antimicrobial activity of Coriloxin was investigated against *Staphylococcus aureus, Pseudomonas aerugionsa* and *Candida albicans* with MIC values of 100 μg /ml, 100 μg /ml and 200 μg /ml respectively. Coriloxin was also found to inhibit root growth of lettuce to 46% at a concentration of 50 μg/ml [2].

Coriloxin has been isolated previously at Bradford University from X. *Obovata* [3] and X. *Badia* [4]. Adeboya [3], who reported the investigation of the absolute

stereochemistry of coriloxin at the epoxy group(C5-C6) and C4 using Cotton curves, suggested the structure shown in Figure 71.

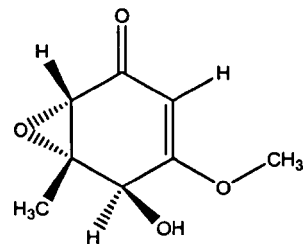


Figure 71 Coriloxin structure by Adeboya

Yoshihito *et al* [2] in 2005 also reported the investigation of the absolute stereochemistry of coriloxin at the epoxy group (C5-C6) and C4 using the same method and suggested the structure shown in Figure 72.

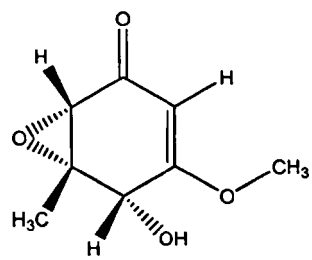


Figure 72 Coriloxin structure by Yoshihito *et al.*

In order to resolve this confusion, X-Ray crystallography (Figure 73) has been used during the current investigation to determine the stereochemistry of coriloxin. The XRD data confirms that the correct configuration is that proposed by Yoshihito *et al.*

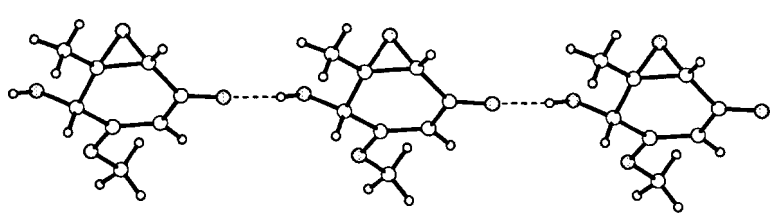


Figure 73 X-Ray crystallography of coriloxin

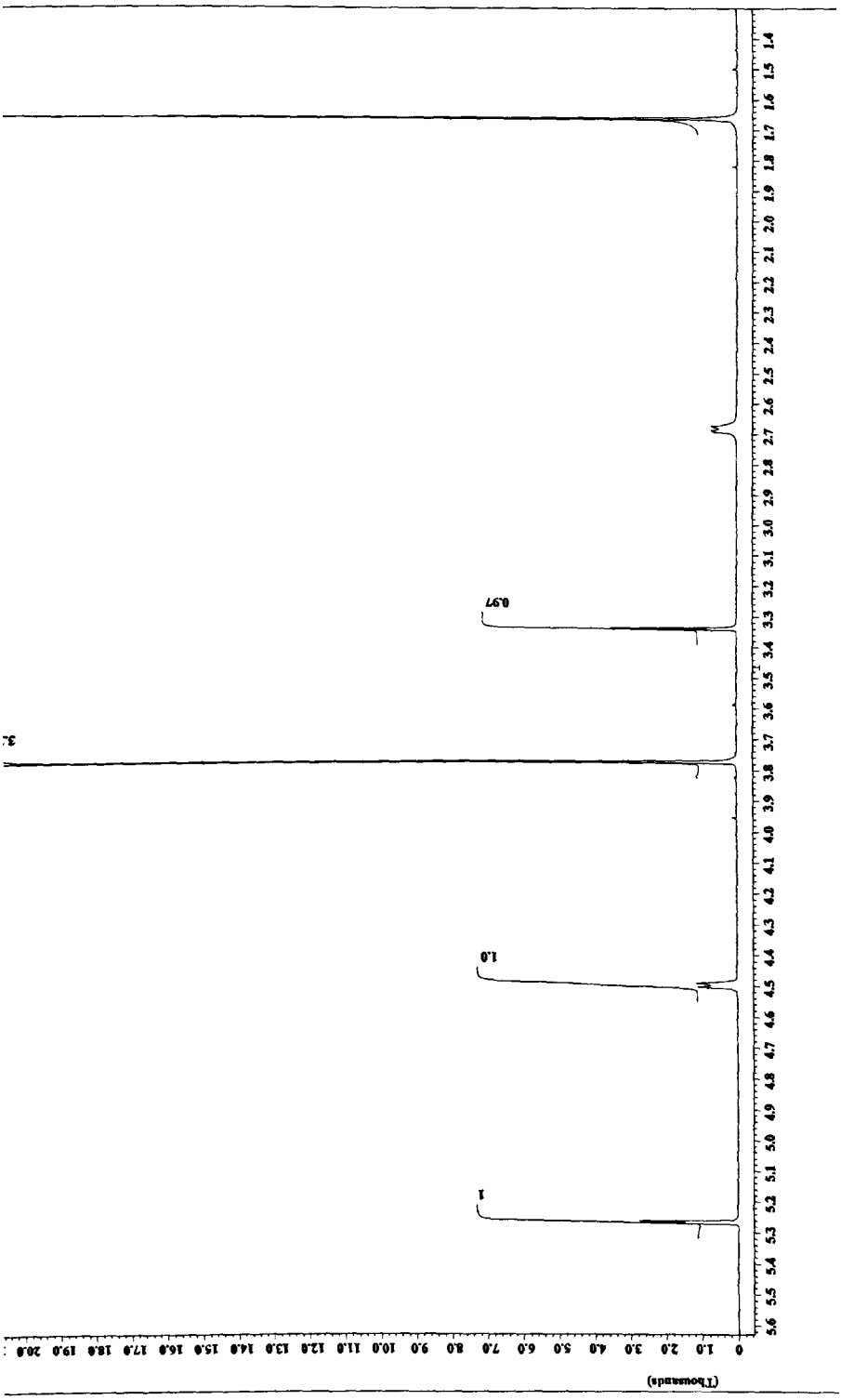


Figure 74 ^{1}H NMR spectrum of coriloxin

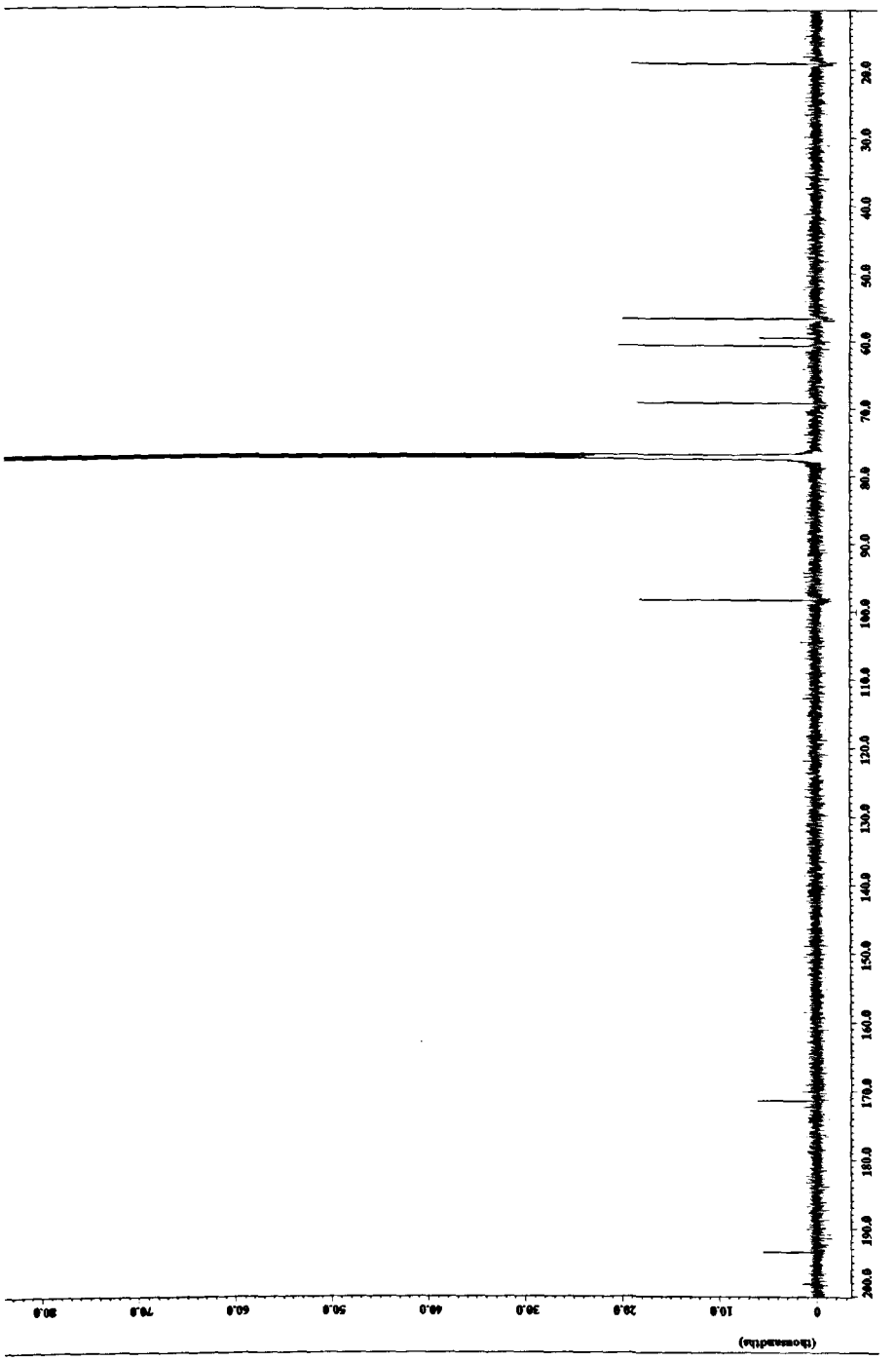


Figure 75 ^{13}C NMR spectrum of coriloxin

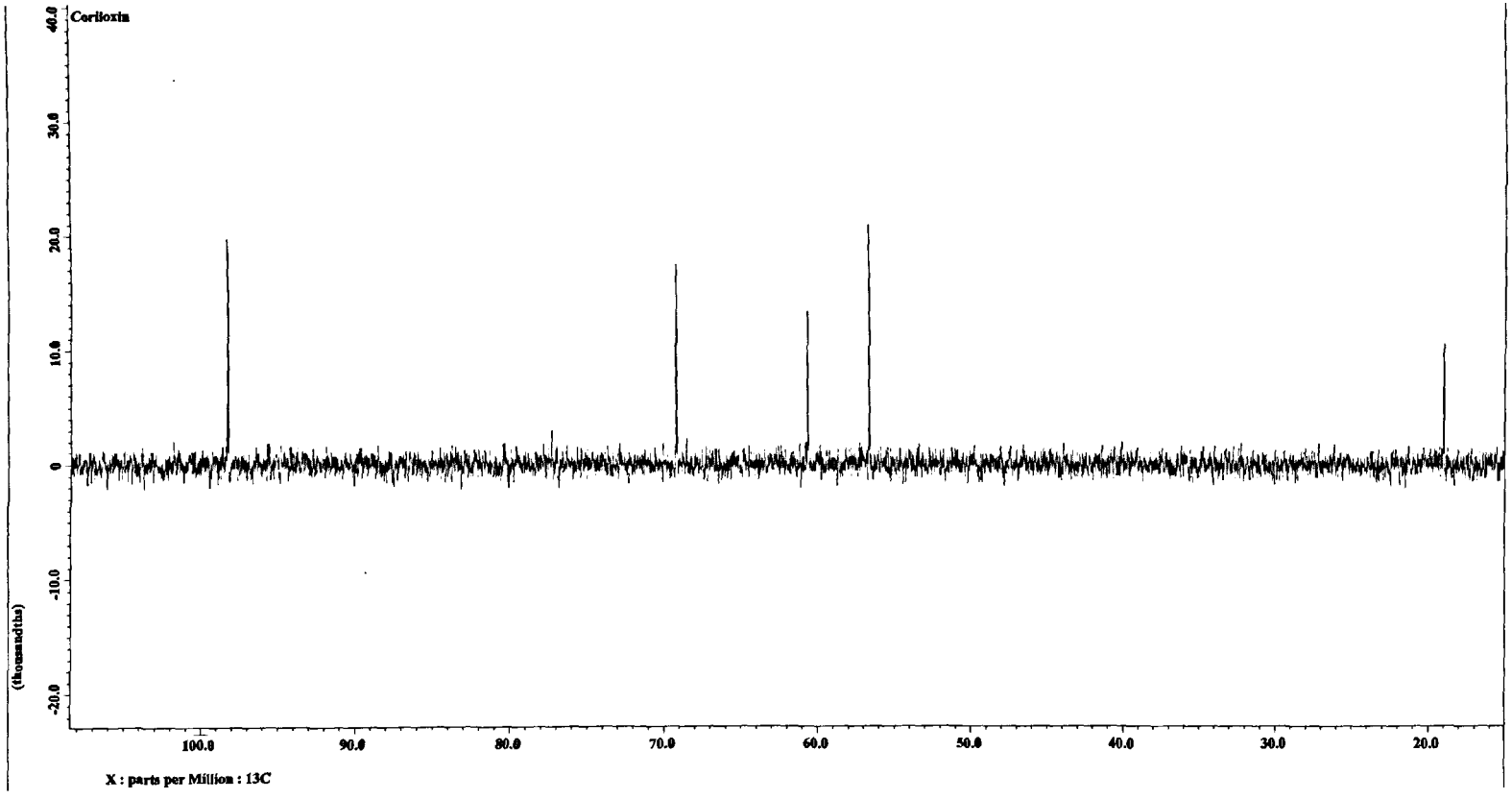


Figure 76 DEPT-135 NMR spectrum of coriloxin

5.2 Isolation of mellein from X04

The ethyl acetate extract (4.3 g), which remained after removal of coriloxin, was chromatographed over a silica gel column (60 × 2.5 cm). The column was eluted with a mixed solvent system [petroleum ether (60-80 °C), ethyl acetate and acetic acid (50:50:1)] and fractions of 3.0 ml were collected using an automated fraction collector.

The first component recovered (tubes 74-83) was yellowish oil, which was treated with *n*-hexane to give a white solid (8 mg), which was recrystallised from the same solvent to give white plates (3 mg) , mp 52-55 °C; ES $[M+H]^+$ *m/z* 179; $[\alpha]_D^{23}$ -117° (c 0.05, MeOH). In the TLC plate eluted with petroleum ether (60-80 °C), ethyl acetate and acetic acid (50:50:1) or toluene, ethyl acetate and acetic acid (50:49:1) this compound had a R_f value of 0.92. It also showed a strong orange spot with *p*-nitroaniline spray reagent, but was not active with anisaldehyde spray reagent.

The ^1H NMR spectrum ($CDCl_3$) shows ten protons with signals at δ_H 1.51 (3H, d, *J* 6.42 Hz), 2.91 (2H, d, *J* 6.88 Hz), 4.71 (1H, m), 6.67 (1H, d, *J* 7.34 Hz), 6.87 (1H, d, *J* 8.25 Hz), 7.39 (1H, dd, *J* 7.39 and 8.42 Hz), 11.01 (1H, s). The 3 sets of signals in the aromatic region indicated a tri-substituted ring. The signal at δ_H 11.01 suggests a chelated hydroxyl group. The signals at δ_H 1.51, 4.71 and 2.91 represent a CH_3CHCH_2 subunit.

In the ^{13}C NMR spectrum run in $CDCl_3$ ten carbon signals comprise a methyl at δ_C 20.88, a methylene at 34.70, four methine carbons at 76.24, 116.32, 118.04, and 136.27, and four quaternaries at 108.38, 139.52, 162.27, and 170.09. The mass spectrum, ^1H and ^{13}C NMR data, suggested a molecular formula of $C_{10}H_{10}O_3$.

Comparison of the spectral data, mp and the optical rotation of this compound with reported data identified this compound as R-mellein (Figure 77) [5-8]

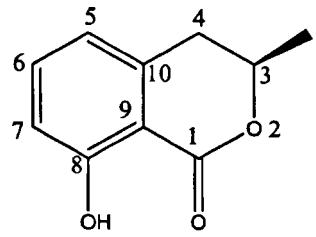


Figure 77 R-Mellein structure

^{1}H, ^{13}C and HMQC NMR data of mellein is given in Table 19. The structure elucidation of mellein is described in more detail in chapter 4.

Table 19 ^{1}H and ^{13}C NMR data ($CDCl_3$) of R-mellein

No	δ_C	δ_H
1	170.09 – C	
3	76.24 – CH	4.71 (1H, m)
4	34.70 – CH_2	2.91 (2H, d, *J* 6.88)
5	118.04 – CH	6.85 (1H, d, *J* 8.25)
6	136.27 – CH	7.39 (1H, dd, *J* 7.33, 8.40)
7	116.32 – CH	6.67 (1H, d, *J* 7.34)
8	162.27 – C	
9	108.38 – C	
10	139.52 – C	
11	20.88 – CH_3	1.51 (3H, d, *J* 6.20 Hz)
*	Chelated OH	11.01 (1H, s)

The first isolation of this compound was in 1933 from Aspergillus *melleus* [6-7]. It has also been isolated at Bradford from different Xylaria fungi such as X.*Longiana* [8], X.*grammica* [9] and X.*Badia* [4]. In this project S-mellein has been found to be produced by the xylaria fungi A217R and A517R described in chapter 4. which

means that this metabolite is a common secondary metabolite for xylaria fungi. The yield of mellein from most of the fungi is very low compared to other metabolites. It has also been found that fungi, which produce this metabolite, can produce other derivatives such as 4-hydroxymellein and 8-methoxymellein.

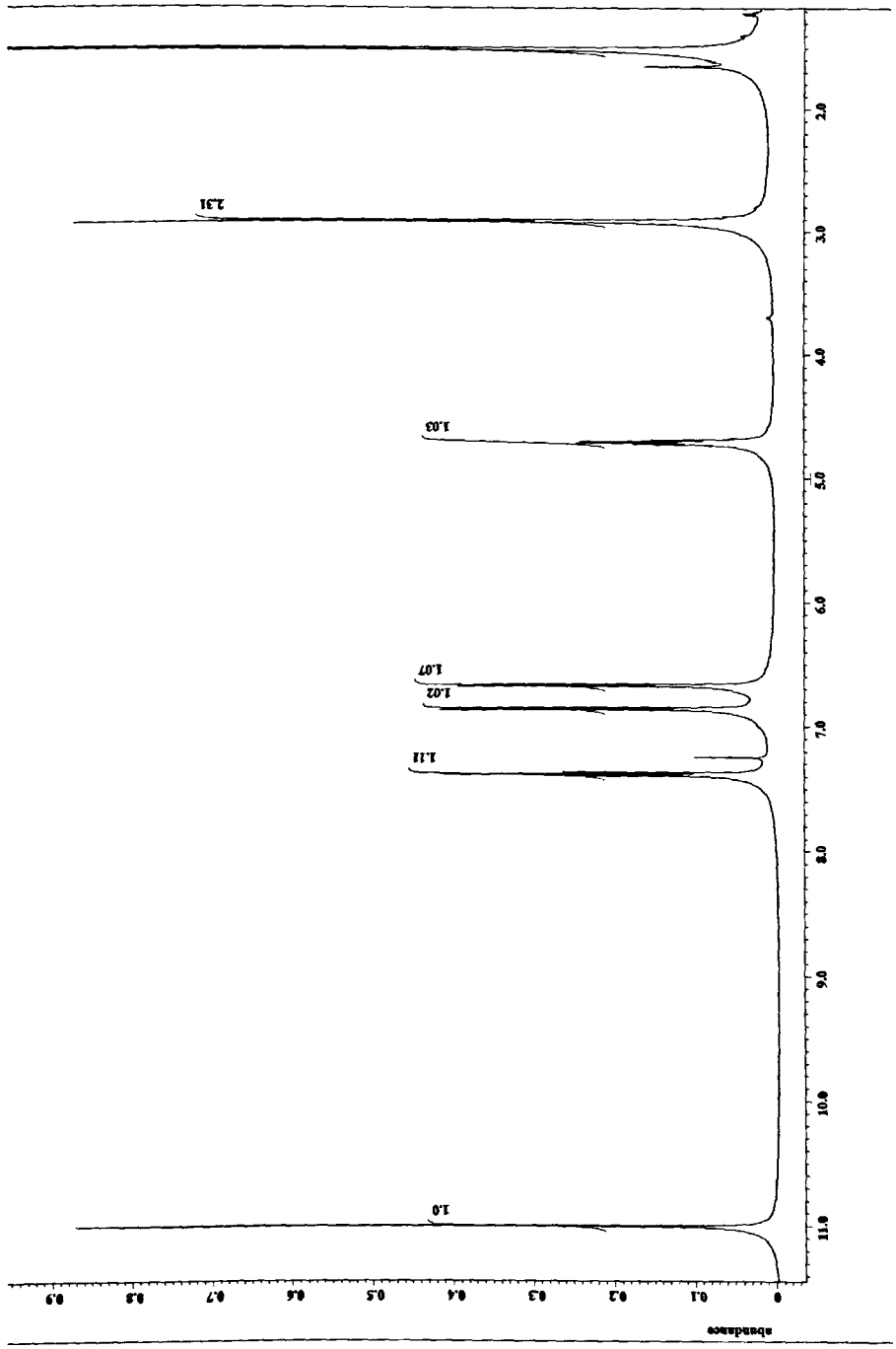


Figure 78 ^{1}H NMR spectrum of mellein from X04

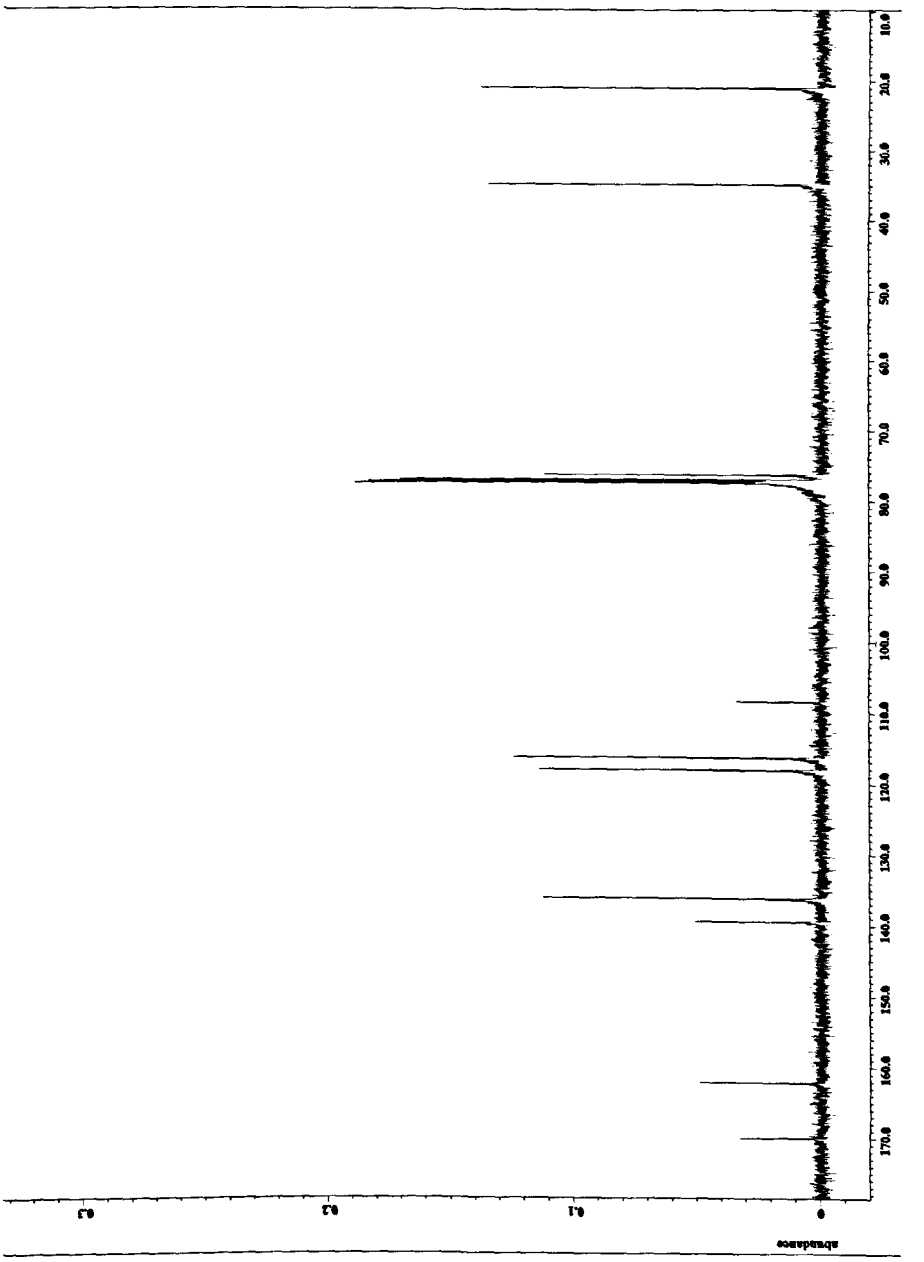


Figure 79 ^{13}C NMR spectrum of mellein from X04

5.3 Isolation of stereoisomeric mixture of 4-Hydroxymellein

The second component collected from the column (tubes 85-100) had an R_f value of 0.83 and gave orange spot with *p*-nitroaniline spray reagent. It was isolated as white solid and recrystallised from hot petroleum as fine white needles, mp 119-122 °C; *m/z* 195 $[M + H]^+$ in its ES mass spectrum.

The ^1H and ^{13}C NMR spectra (Figure 81 and Figure 82) suggested a mellein derivative, which exists as a mixture of two isomeric forms in unequal ratio. The C-4 methylene signals of mellein are absent and have been replaced by an oxygenated methine signal at δ_H 4.56/4.58 and δ_C 69.91/67.29. Replacement of the methylene group with a hydroxy-methine group at position 4 in mellein molecule gives 4-hydroxymellein ($C_{10}H_{10}O_4$).

Comparison of the spectral data for this compound with reported data for 4-hydroxymellein (Table 20) confirms this compound as 4-hydroxymellein (Figure 80)

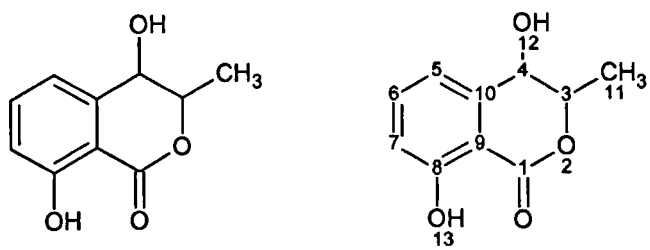


Figure 80 4-Hydroxymellein

Table 20 NMR data for 4-Hydroxymellein in $CDCl_3$

Experimental results				From X. *Longiana* [10]	
NO	δ_C	DEPT	δ_H (mult)	δ_C	δ_H (mult)
1	168.59	C		169.24	
1	169.29	C		169.32	
3	78.30	CH	4.68 qd	78.30	4.61 qd
3	80.06	CH	4.60 m	80.02	4.70 q
4	67.29	CH	4.56 m	67.19	4.61 d
4	69.19	CH	4.58 m	69.06	4.70 d
5	118.39	CH	6.91 d	118.38	6.95 d
5	118.60	CH	7.00 m	118.48	7.01 dd
6	136.89	CH	7.51 qd	136.83	7.53 qd
6	136.97	CH	7.52 qd	136.90	7.55 qd
7	116.35	CH	7.01 m	116.36	7.01 dd
7	117.87	CH	6.97 m	117.75	6.95 d
8	162.05	C		161.94	
8	162.14	C		162.02	
9	106.73	C		106.66	
9	106.91	C		106.85	
10	140.58	C		140.54	
10	141.27	C		141.27	
11	16.09	CH_3	1.56 (d)	16.02	1.52 (d)
11	18.00	CH_3	1.50 (d)	17.94	1.60 (d)
*	O-H		10.98 (s)		10.99 (s)
*			10.95 (s)		11.01 (s)

The ^1H NMR spectrum ($CDCl_3$) of the isomeric mixture of *cis*- and *trans*-4-hydroxymellein exhibited 6 down-field signals at δ_H 6.91, 6.97, 7.00, 7.01, 7.51 and

7.52 and six up-field signals at δ_H 1.50, 1.56, 4.56, 4.58, 4.60 and 4.68 as well as two highly deshielded singlet at δ_H 10.98 and 10.95).

The *cis*-isomer was characterized by the presence of three downfield signals for aromatic protons in the ^{1}H NMR, which appeared as one-proton doublets at 6.91 (J = 7.0 Hz) and 7.01 (J = 7.0 Hz), a one-proton triplet at 7.51 (J = 7.0 Hz), a three-proton at δ_H 1.56 (d, J = 6.5 Hz) for a methyl group, a one-proton doublet of a quartet at δ_H 4.68 (J = 2.06, 6.7 Hz) as well as a one-proton broad singlet at 2.5. Furthermore, a hydrogen-bonded phenolic proton appeared as a highly deshielded one-proton singlet at 10.98. The *cis*-configuration was assigned to H-3 and H-4 on the basis of the small coupling constant (2.04 Hz) whereas the analogous *trans*-isomer had a higher value 4.5 Hz [11]. This compound was isolated before in Bradford from X. *longiana* [10] and X. *Grammica* [9]. However the metabolite profile of this fungus is different from above species.

Cis-4-hydroxymellein was previously isolated from several fungi, including *Lasiodiplodia theobromae* [11], *Cercospora taiwanensis* [12], *Aspergillus ochraceus* [13] and *Aspergillus melleus* [14] while the *trans* isomer was reported from *Apiospora camptospora* [11], *Septoria nodorum* [15]. In this project, the pure *cis*-3S,4S-4-hydroxymellien was isolated from the fungus A217R and A517R described in chapter 4.

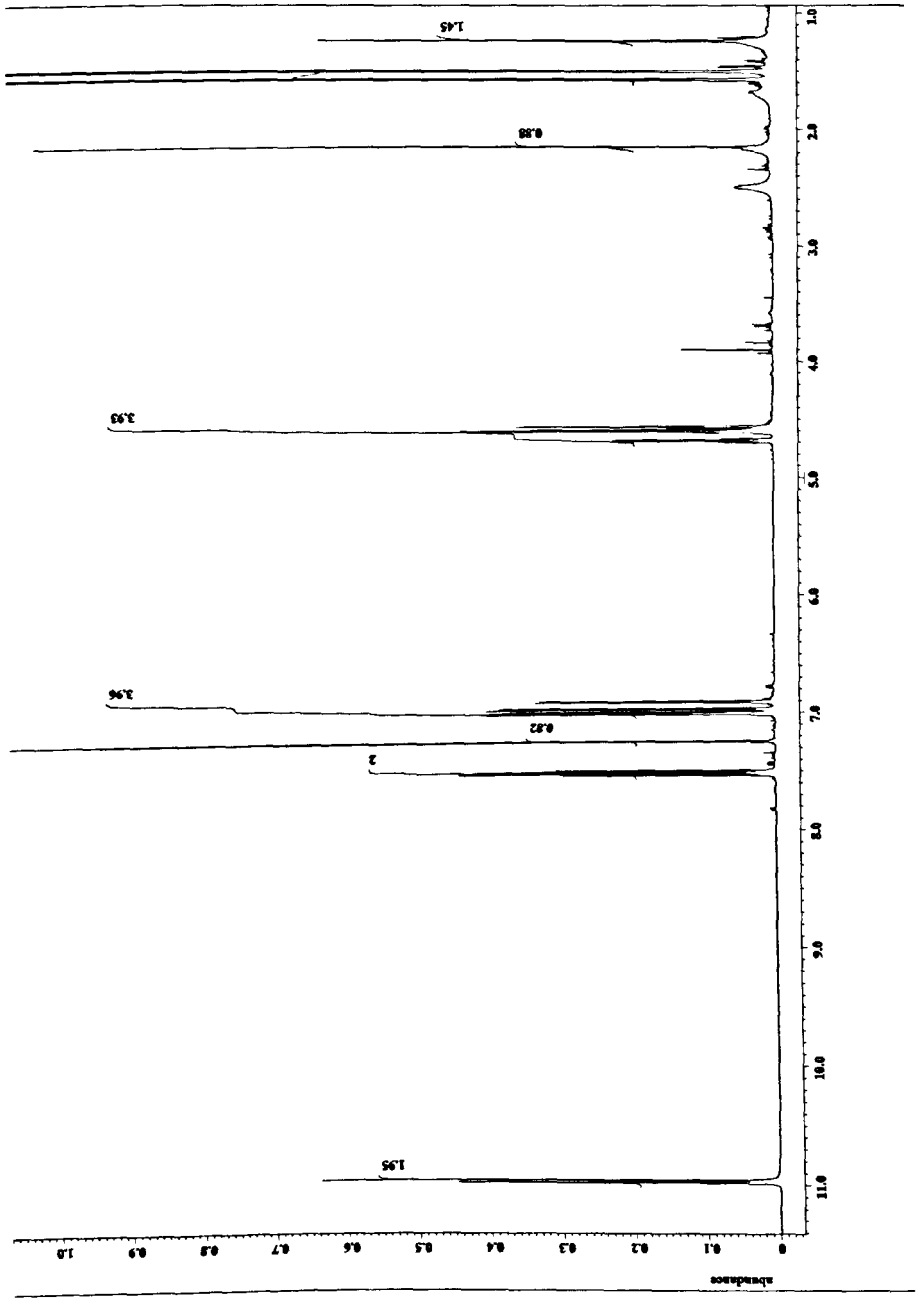


Figure 81 ^{1}H NMR spectrum of 4-Hydroxymellein

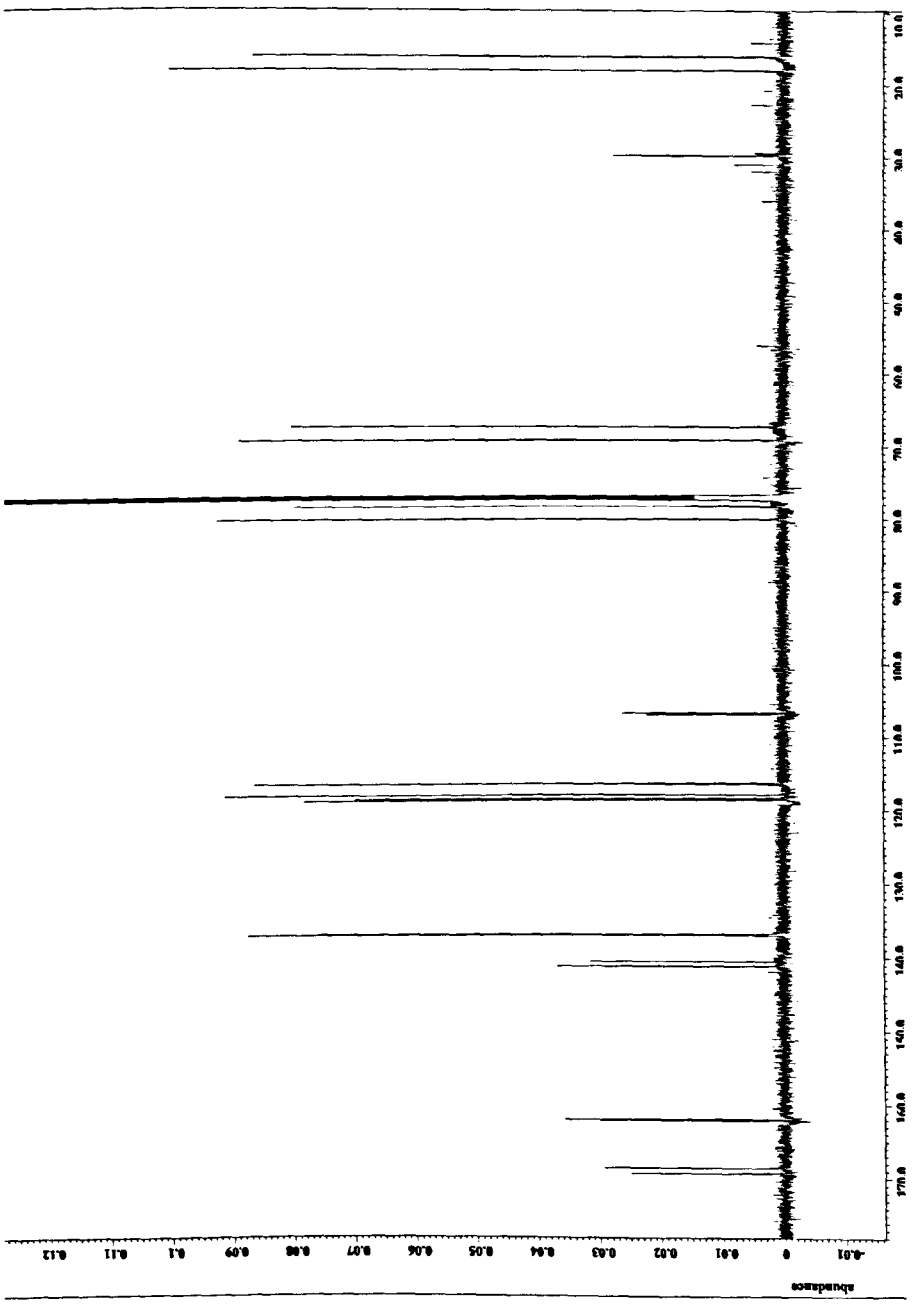


Figure 82 ^{13}C NMR spectrum of 4-Hydroxymellein

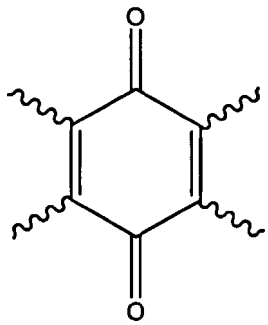
5.4 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

The third component from the column chromatography (Tubes 110-135) was a mixture of two compounds. The main component gave a violet colour on the silica gel TLC plate at R_f 0.45 after a few hours exposure to air and light. Three 20 × 20 cm silica gel PLC plates were used to separate 300 mg of the mixture. The violet band was recovered and washed with ethyl acetate to give orange crystals after the evaporation of the solvent. Recrystallisation from petroleum ether (80-100 °C) gave orange crystals (8.7 mg), mp 117-122 °C, ES $[M+H]^+$ *m/z* 183. The $IR_{(KBr)}$ spectrum showed the an absorption at 3348 cm^{-1}, suggesting an OH group, 1640 cm^{-1} a conjugated carbonyl group and 1598 cm^{-1}, which may be due to a conjugated double bond.

A ^1H NMR spectrum determined in $CDCl_3$ (Figure 86) revealed 2 methyl signals at δ_H 1.49 (3H, t) and 1.93 (3H, s) methylene protons at δ_H 4.03 (2H, q), a singlet proton at δ_H 7.28 (1H, s) and a methine proton at δ_H 5.79 (1H, s), which could be an olefinic proton.

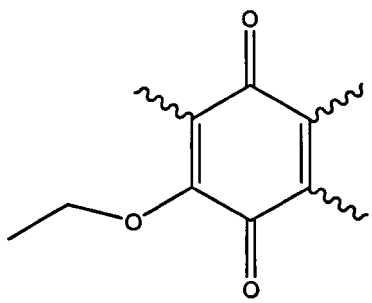
^{13}C NMR spectrum in $CDCl_3$ (Figure 87) and DEPT-135 NMR spectrum (Figure 88) revealed 9 signals comprising two methyl groups at δ_c 7.98 and 13.92, one methylene carbon signal at 65.96, a methine carbon at δ_C 102.51 and five quaternary carbons at δ_C 114.88, 151.61, 160.57, 182.22 and 182.85. The last two carbon signals suggested conjugated ketone carbonyl groups.

The *m/z* 183 $[M+H]^+$ combined with the NMR spectral data indicated a molecular formula $C_9H_{10}O_4$ with 2 carbonyl groups conjugated with double bonds. This formula requires 5 degrees of unsaturation, which could be due to 2 carbonyl groups, 2 double bonds and a ring; such as substructure **A**.



A

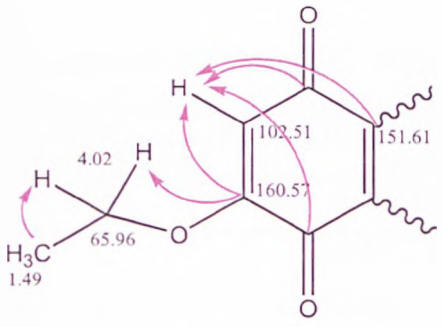
The downfield methylene signal at δ_c 65.96, which correlates to δ_H 4.03 (q) in the HMQC NMR spectrum (Figure 89), indicated it is adjacent to an oxygen atom. The proton multiplicity suggested it was adjacent to a methyl group, which must be the one at δ_H 1.49 (3H, t), which showed a cross peak with δ_c 13.92. Adding the above information to substructure **A** gives substructure **B**.



B

The ^{1}H-^{1}H COSY spectrum wasn't helpful in the assignment of the connectivity of the rest of the molecule. HMBC correlations were used to establish the structure. In the HMBC NMR spectrum (Figure 90), the methylene protons at δ_H 4.02 showed a cross peak with δ_C 160.57 which in turn showed a cross peak with the vinylic proton at δ_H 5.79, which correlates to the carbon at δ_C 102.51 in the HMQC NMR spectrum.

The vinylic proton also showed 2 more HMBC cross peaks with δ_C 182.22 and 151.61 to give substructure **C**.



C

The δ_C 151.61 showed more HMBC correlations with the protons at δ_H 1.93, which has a correlation with δ_C 7.98 in the HMQC NMR spectrum, and δ_H 7.25, which didn't show any correlation in the HMQC NMR spectrum (Figure 89) and it was considered as a hydroxyl proton. By adding the last described connectivity to substructure C, the final structure was confirmed (Figure 83 and Figure 84).

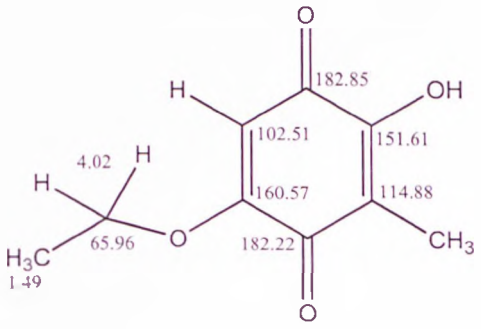


Figure 83 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

ACD/LABS NMR prediction software (v.10) was used to assist the identification of the compound. The ^{1}H and ^{13}C NMR predictors were used to predict the spectra for

the suggested structure of the compound. The calculated values of the chemical shifts were compared with the experimental values as shown in Table 21 and showed good agreement.

Table 21 Experimental and calculated ^{1}H and ^{13}C NMR data for 2-hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

	Experimental results			Calculated values		
C#	δ_C	DEPT	δ_H (mult)	δ_C	DEPT	δ_H (mult)
1	182.85	C		184.33	C	
2	151.61	C		154.66	C	
3	114.88	C		119.20	C	
4	182.22	C		182.52	C	
5	160.57	C		160.39	C	
6	102.51	CH	5.79	102.87	CH	5.89
7	7.98	CH_3	1.93	8.65	CH_3	1.93
8	65.96	CH_2	4.03	65.76	CH_2	4.08
9	13.92	CH_3	1.49	13.92	CH_3	1.42
*		OH	7.25		OH	7.03

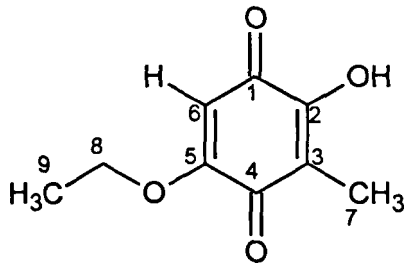


Figure 84 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

A literature search revealed that this compound is novel. A closely related compound (2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione) has been isolated previously at Bradford from *Xylaria badia* [4] (Figure 85.).

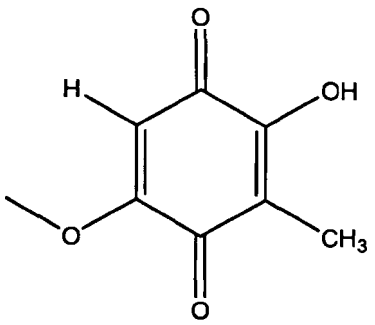


Figure 85 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione

Xylaria badia was found to produce mellein, coriloxin and a naphthol glucoside in addition to the above benzoquinone derivative. Comparison of the metabolite profile of X04 and *Xylaria badia*, suggests that they are closely related species.

Quinones have an important role in the biosynthesis of many important compounds such as vitamin E and coenzyme Q [16-17].

5.5 Anti-malarial test

Coriloxin, cytochalasin D, mellein and 4-hydroxymellein were tested as anti-plasmodium agent against *Plasmodium falciparum* strain K1. The test was done in The Bradford School of Pharmacy, University of Bradford by Dr Colin W. Wright's research group. Coriloxin gave IC_{50} = 2.22 μg/mL (mean of two experiments), whilst the other compounds showed no activity at the highest dose tested (50 μg/mL).

Gessler *et al.*[18] suggested that very good antiplasmodial acitivity extract should displayed an IC_{50} less than 10 μg/ml. Moderate activety for IC_{50} from 10 to 50 μg/ml and over 50 μg/ml the extract was considered to have low activity.

According to criteria suggested by Deharo *et al.*[19], an extract can be considered as good activety when its IC_{50} is less than 5 μg/mL, moderately active when its IC_{50} is between 5 and 10 μg/mL and inactive when its IC_{50} is over 10 μg/mL.

Based on the last criteria, Coriloxin with IC_{50} = 2.22 μg/mL can be concluded as having a good antiplasmodial activity, whilst the other compounds showed no activity at the highest dose tested (50 μg/mL).

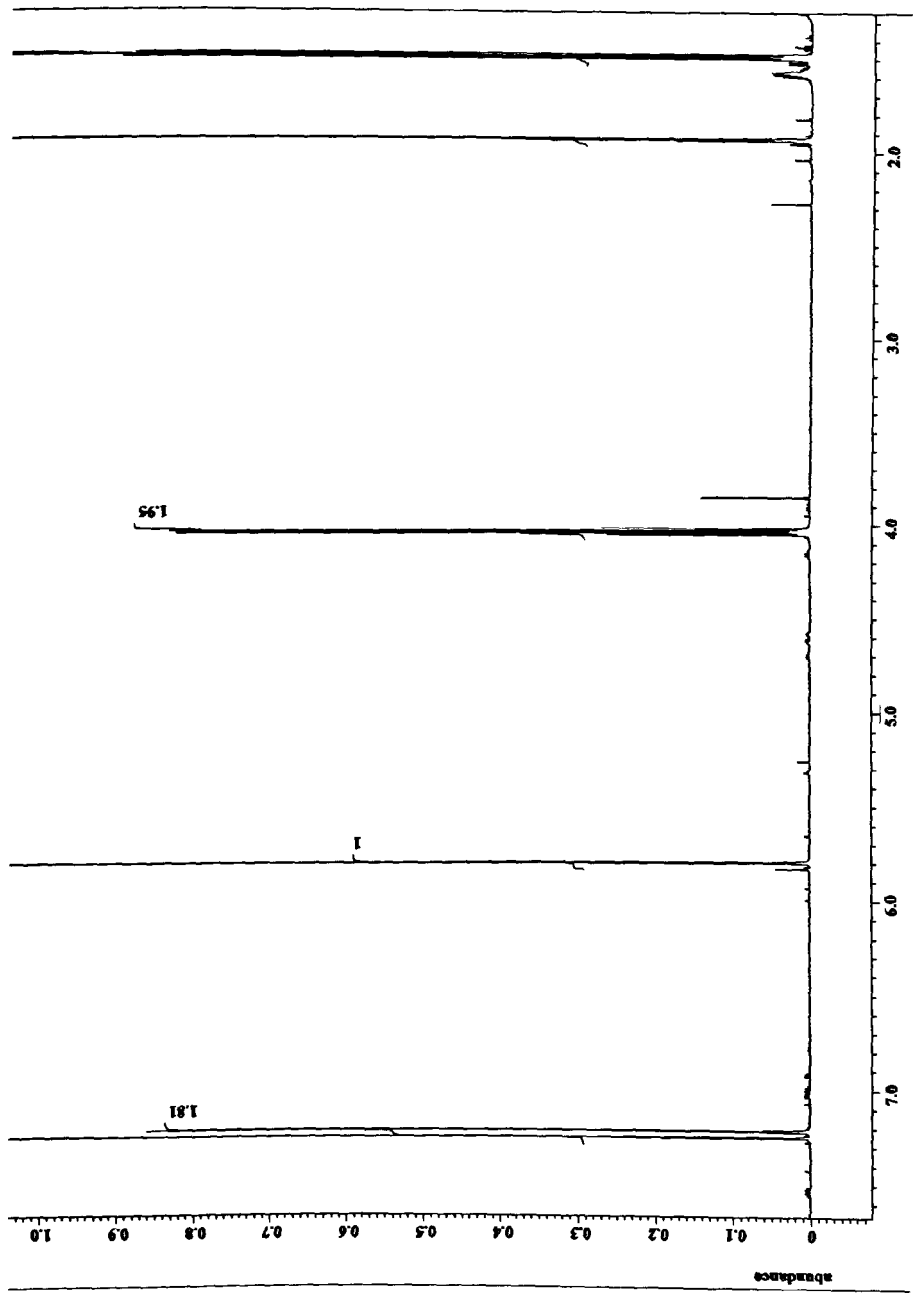


Figure 86 ^{1}H NMR spectrum of 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

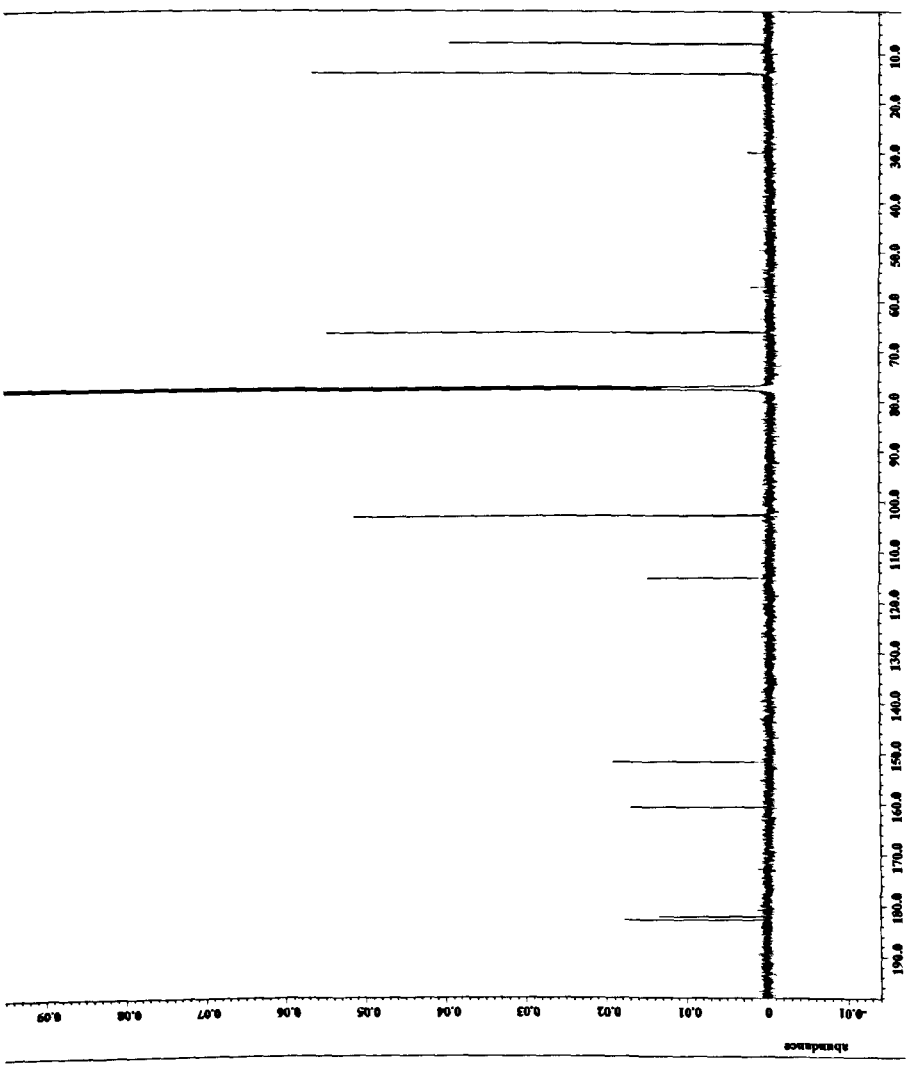


Figure 87 ^{13}C NMR spectrum for 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

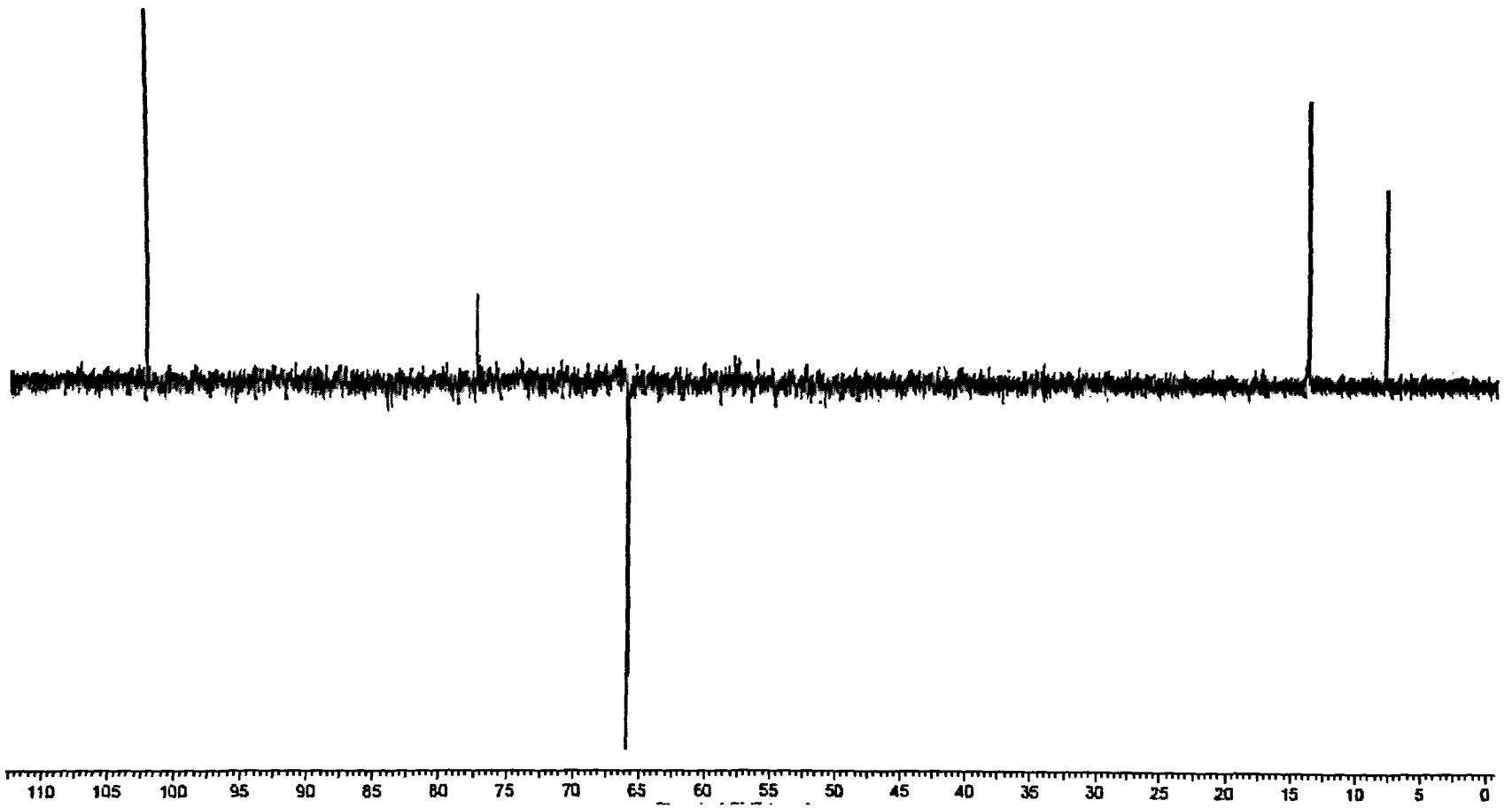


Figure 88 DEPT135 spectrum for 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

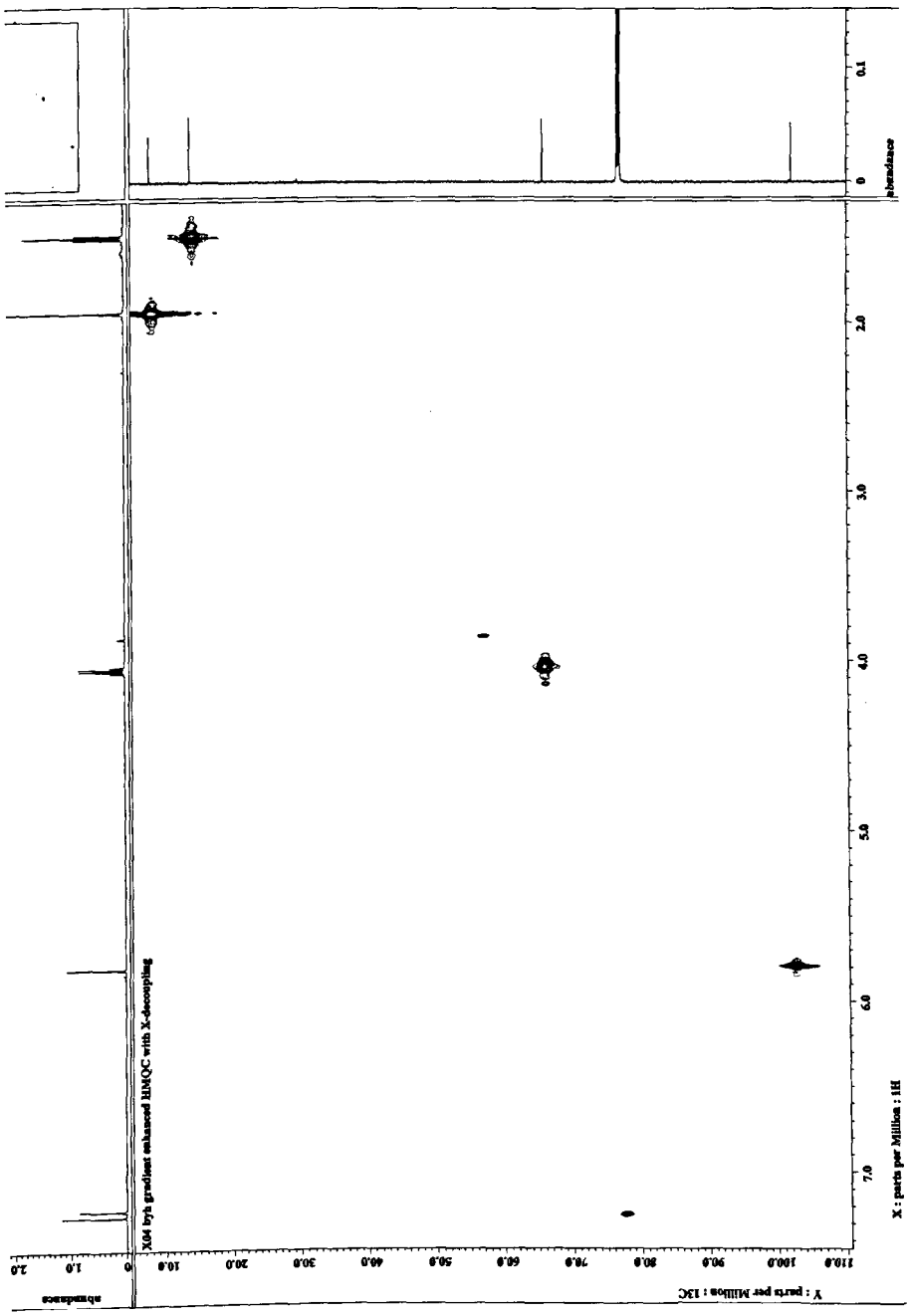


Figure 89 HMQC spectrum for 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

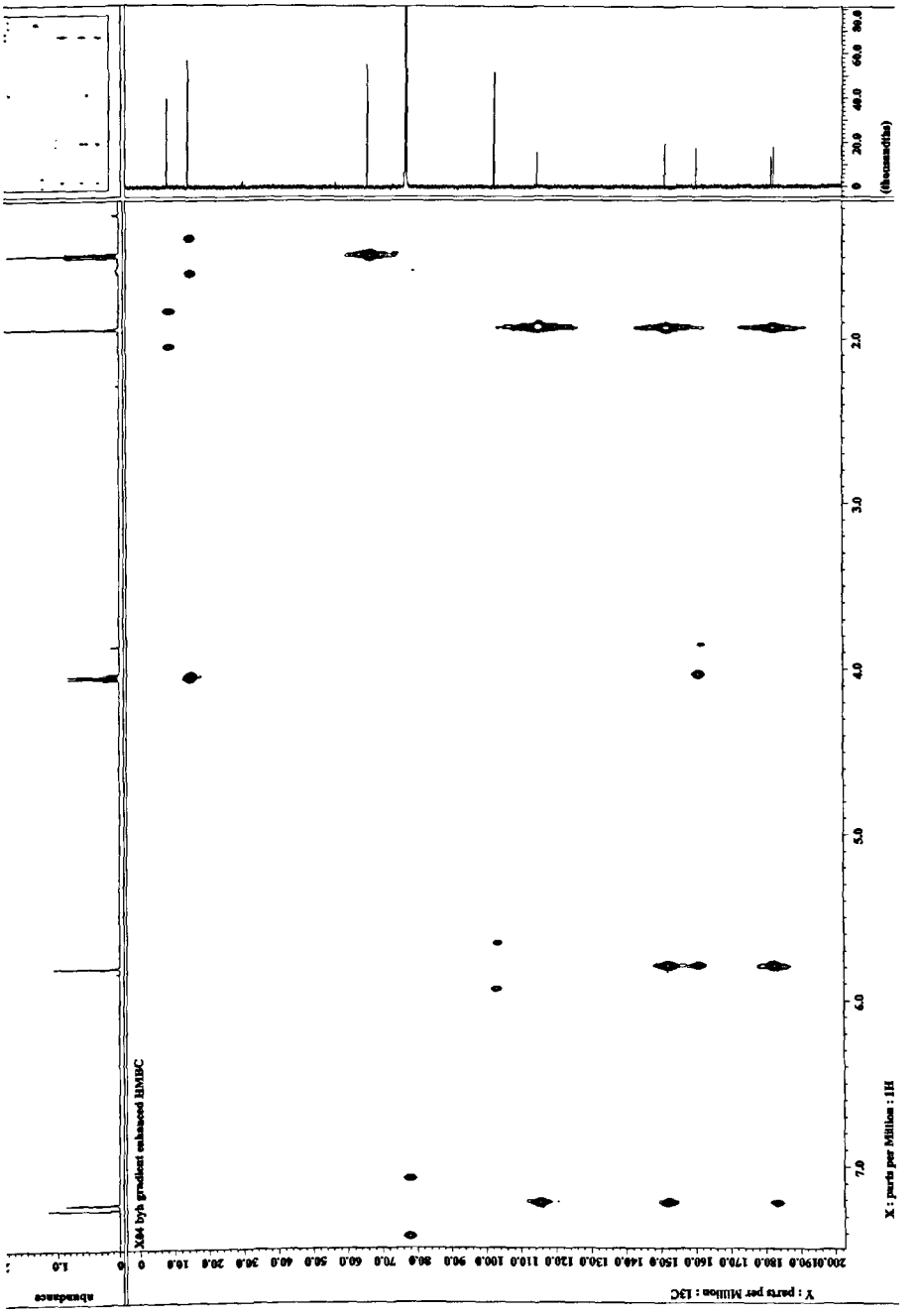


Figure 90 HMBC spectrum for 2-Hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione

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Chapter 6 Secondary metabolites from fungus X.6RD8

6.1 8-O-Methylmellien

The fungus 6RD8 was collected in Thailand. It was cultured on 3% aqueous malt extract mixed with 6% glucose solution for 8 weeks to yield a thin yellowish white mycelium and a brown medium. The ethyl acetate extract of the latter gave a light brown gum (1.7 g). TLC examination of this gum using the solvent system toluene: ethyl acetate: acetic acid (50:49:1) showed 3 main metabolites, which were detected as UV active compounds and as yellowish orange spots when sprayed with *p*-nitroaniline spray reagent.

Trituration of the gum with light petroleum (60-80 °C) afforded white crystals, which were crystallised from the same solvent to give white cubic crystals. After several days the remaining gum began to solidify as needle-like crystals. The solidified crystals were recrystallized from light petroleum ether (60-80 °C) to afford similar cubic crystals, (65.5 mg). mp 84-87 °C; ES $[M+H]^+$ *m/z* 193 ($C_{11}H_{10}O_3$); $[\alpha]_D^{22}$ + 210 (c 1.0, MeOH), $IR_{(ATR)}$ ν_{max} cm^{-1} 2918, 1709, 1596.

The ^1H NMR spectrum ($CDCl_3$, Figure 92) revealed a methyl group at δ_H 1.45 (3H, d, *J* 6.42 Hz), a methoxy group at δ_H 3.92 (3H, s), a methylene signal at δ_H 2.84 (2H, m), methine proton at δ_H 4.53 (1H, m), aromatic protons at δ_H 6.76 (1H, d, *J* 7.34 Hz), 6.89 (1H, d, *J* 8.25 Hz) and 7.43 (1H, dd, *J* 7.57, 8.43 Hz).

The ^{13}C NMR spectrum ($CDCl_3$, Figure 93) and DEPT-135 (Figure 94) showed 11 signals at δ_C 20.79 (CH_3), 36.19 (CH_2), 56.27 (CH_3), 74.22 (CH), 110.98 (CH), 113.74 (C), 119.29 (CH), 134.55 (CH), 142.08 (C), 161.27 (C) and 162.86 (C).

The ^1H NMR spectrum (Figure 92) shows that this compound is very similar to mellein except for the absence of the chelated hydroxyl signal at δ_C 11.05 and the presence of a methoxy signal at δ_C 3.95 (Table 22). Replacement of the chelated hydroxyl group with a methoxy group suggests 8-O-methylmellein. The IR spectrum of this compound showed the carbonyl absorption at 1709 cm^{-1} whilst that of mellein is appearing at 1662. This can be explained to the hydrogen bond effect in mellein. Comparison of the spectral and physical data of this compound with the reported data for 8-*O*-methylmellein confirmed the suggestion [1,2], that the metabolite is (S)-(+)-8-*O*-methylmellein (Figure 91).

Table 22 NMR data ($CDCl_3$) of 8-*O*-methylmellein and mellein

	8-O-methylmellien		**Mellein**	
No	δ_C	δ_H	δ_C	δ_H
1	162.86		170.09	
3	74.22	4.53	76.24	4.74
4	36.19	2.84	34.70	2.94
5	110.98	6.89	118.04	6.90
6	134.55	7.43	136.27	7.41
7	119.29	6.76	116.32	6.70
8	161.27		162.27	
9	113.75		108.38	
10	142.08		139.52	
11	20.79	1.45	20.88	1.54
12	56.27	3.92	Chelated OH	11.05

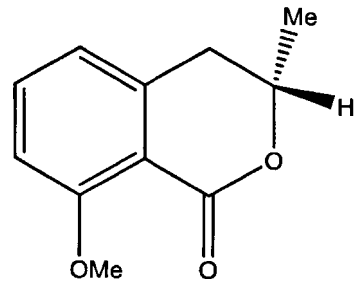

Figure 91 Structure of S-8-*O*-methylmellein

R-8-*O*-Methylmellein was isolated for the first time by Devys *et al* from *septoria nodorum* [1]. The total synthesis has also been reported [3-5]. Dimitriadis *et al* reported the optical rotation of the S-8-*O*-methylmellein and R-8-*O*-methylmellein as $[\alpha]_D^{25}$ +261° and – 250° (c 0.5, $CHCl_3$) respectively [4]. Islam *et al* reported the optical rotation of R-8-*O*-methylmellein as $[\alpha]_D^{21}$ - 252° (c 0.55, $CHCl_3$) [6].

S-8-*O*-Methylmellein was isolated for the first time in University of Bradford from undefined *xylaria* speicies ($[\alpha]_D^{25}$ +238° (c 1.02, $CHCl_3$)) in addition to cytochalasin Q, D, N and O [2]. This the second report of the S-enantiomer from a natural source.

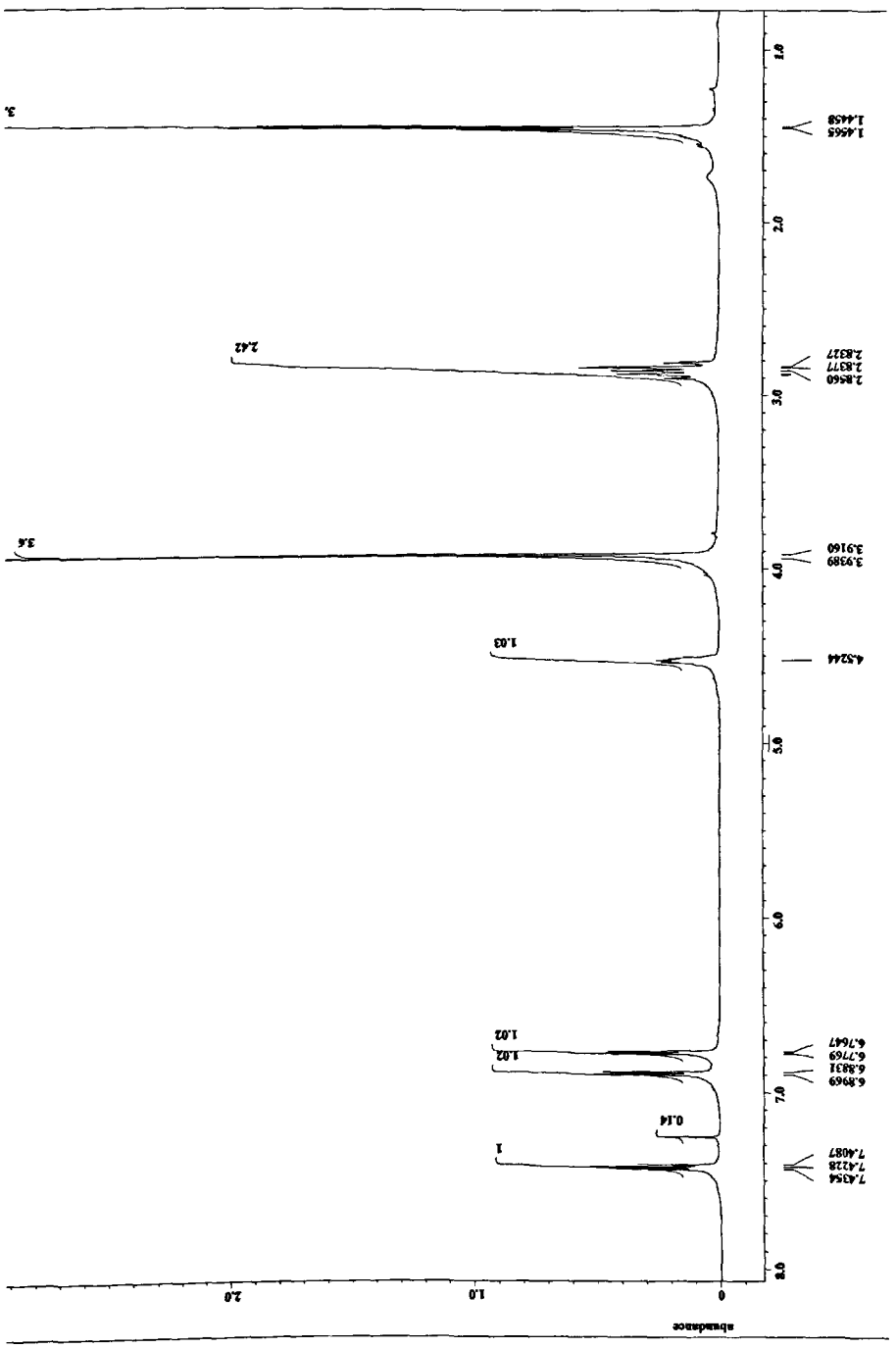

Figure 92 ^{1}H NMR spectrum of S-8-*O*-methylmellein

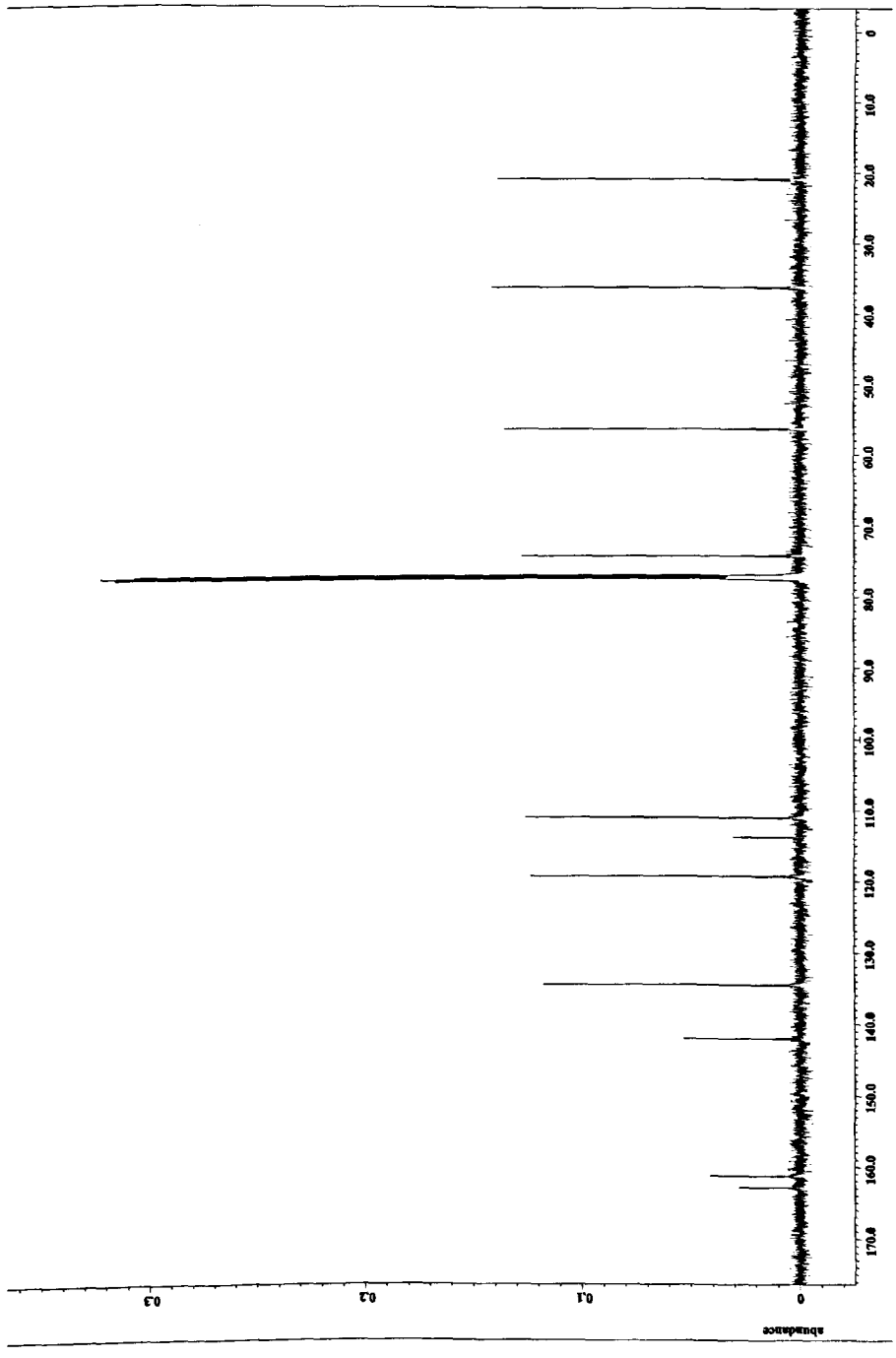

Figure 93 ^{13}C NMR spectrum of S-8-*O*-methylmellein

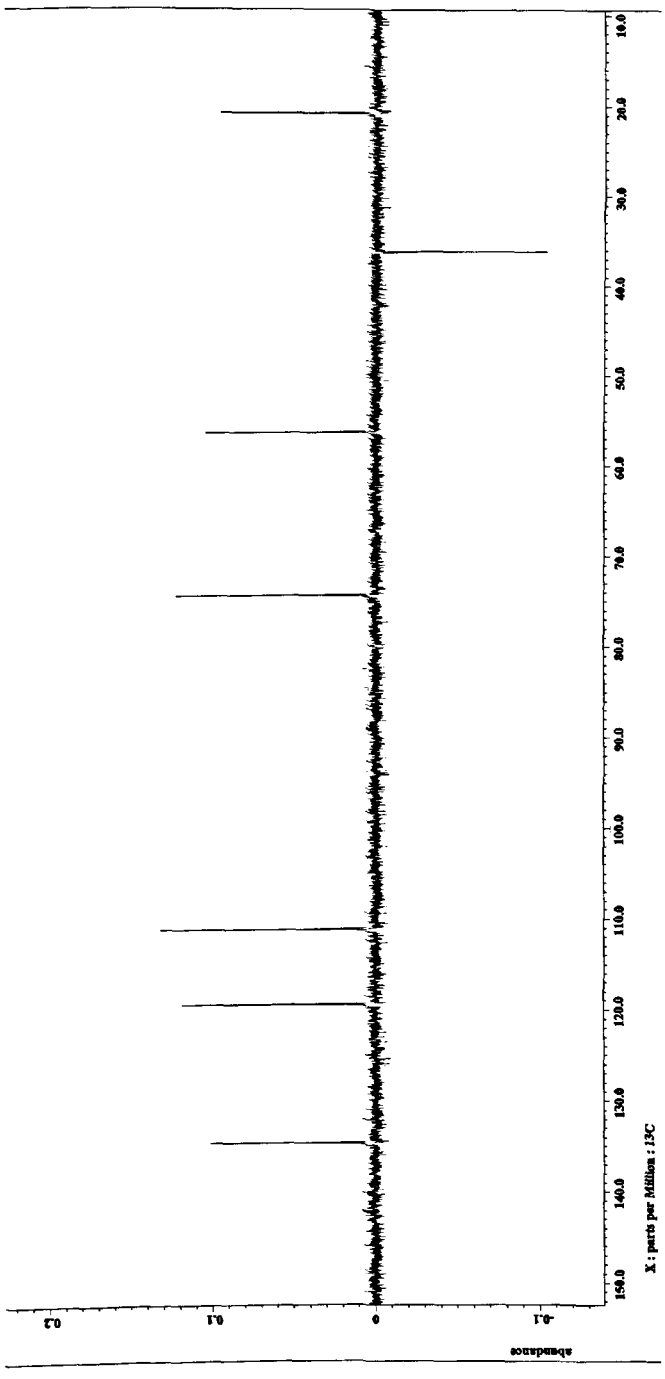

Figure 94. DEPT 135 spectrum of S-8-*O*-methylmellein

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Chapter 7 Experimental

7.1 Culturing of the Fungi

7.1.1 Culture Room

The fungi used as a source of the secondary metabolites were cultured in a special room under specific conditions. The culture room was equipped with following essentials:

- An autoclave for sterilizing culture media before inoculation.
- A Bunsen burner to sterilise glasses and tools such as spatulas used during the culturing process.
- A thermostatic heating system to keep the culture room at stable temperature suitable for fungi growing.
- A thermometer to monitor room temperature.

7.1.2 Glassware inside the culture room

- Thompson bottles (1 L) and (2 L) to grow fungi on large scale.
- Conical flasks (250 ml) to grow the fungi on a small scale.
- Sample tubes with a screw cap to save master cultures of the fungi on agar slopes.

7.1.3 Sterilization

Sterilisation is the process that is used to kill microorganisms such as fungi, bacteria and viruses on a surface, equipment and biological culture media. This process was achieved using a chemical detergent and heating under elevated temperature and pressure as follows:

- First of all, the conical flasks and Thompson bottles were cleaned with water and a biocleaner detergent (2.5 % solution of Microcleanse from Teknon) using a cleaning brush.
- After preparation of the culture medium, a Priorclave was used to complete the sterilisation process of the flasks and Thompson bottles containing the culture medium. The Priorclave uses steam at 121 °C for 30 min. The process is completely automated in this modern autoclave (Figure 95).

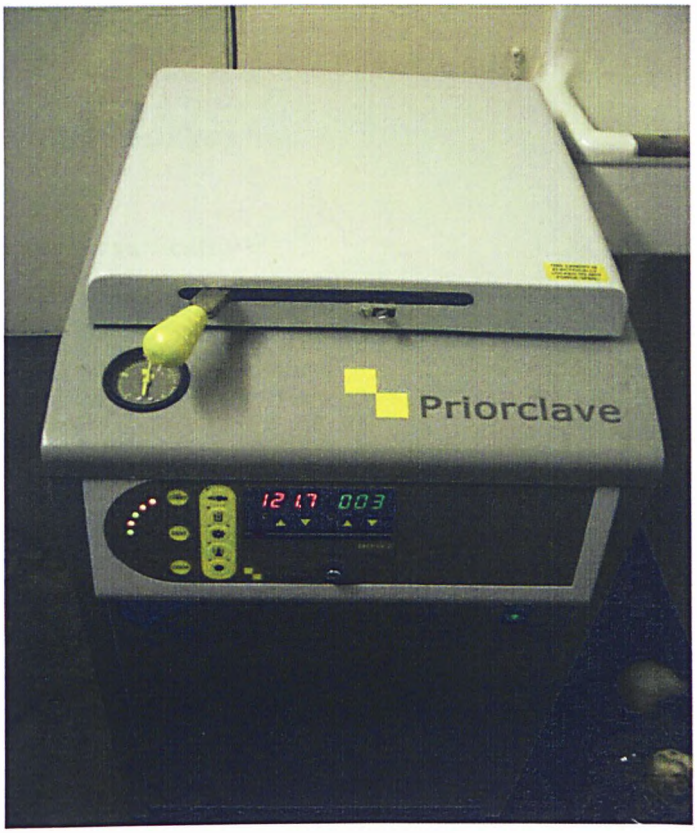


Figure 95 The Priorclave uses steam at 121 °C for 30 min

7.1.4 Master culture

Master fungi samples were kept in 20 ml glass screw-capped tubes. They were prepared as following:

- A suspension mixture of 3% malt and 2% agar was mixed with heating.

- 20 ml sample tubes with cap were filled to approximately half their volumes with the prepared suspension.
- The tubes and contents were sterilised by the Priorclave.
- The tubes then reheated again to 100 °C for 5 min and set in a slope mode so the culture medium solidifies sideway in the tubes.
- Finally the tubes were inoculated with the fungus samples and labelled with code and date.
- For each fungus 4-6 master cultures were prepared. The master cultures are used to sub-culture the fungi into conical flasks, which can be used to reform the master cultures, if required.

7.1.5 Conical flasks sub-culturing:

Each fungus was sub-cultured in 250 ml conical flasks for 2-3 weeks before they transferred into Thompson bottles. These conical flasks provide sufficient material for larger scale culturing. They also give information about the growth pattern of the fungi. The content of each conical flask can be used to inoculate 7-10 Thompson bottles.

7.1.6 Thompson bottles

Thompson bottles were used to culture the fungi for large scale as following:

- A conical flask containing the fungus sample was held horizontally and the cotton wool stopper was removed. The neck of the conical flask was sterilised over a Bunsen burner flame.
- The mycelium of the fungi was crushed into small pieces using a sterilised spatula.

- A small sample of material from the conical flask was added to each Thompson bottle, which contained sterilised culture medium.
- Each set of Thompson bottles was labelled with the fungus code and culturing date and kept on shelves designed for this purpose.

The fungi bottles were observed for 8 weeks and any contaminated bottle was discarded. The culture room was kept dark at 25-27 °C. The culturing process can be repeated several times as required using the master culture. Figure 96 shows the steps in the culturing process.

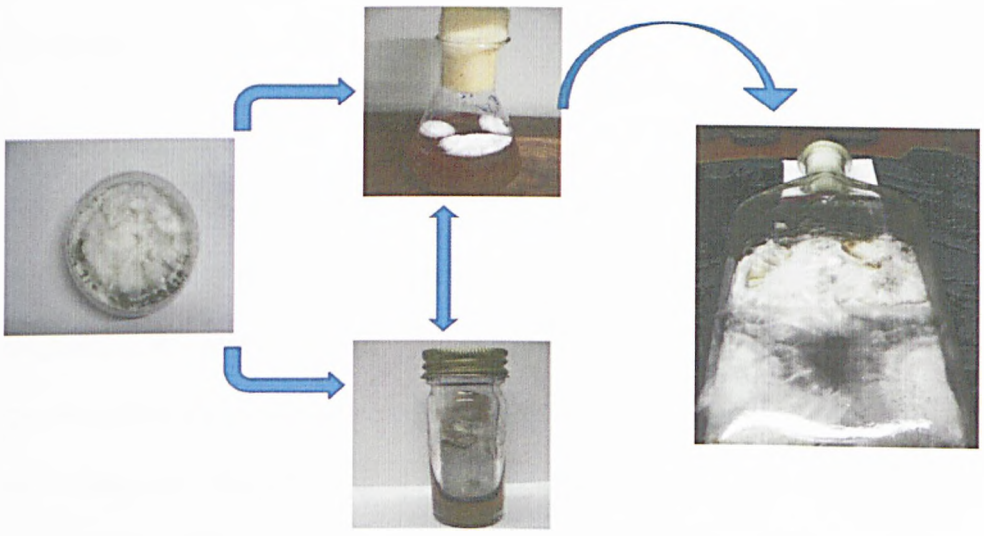


Figure 96 Culturing process steps.

7.2 Extraction process

After eight weeks the growing fungi were combined and the mycelium was separated by filtration through a muslin cloth. The mycelium was air-dried for two weeks. The medium was extracted in batches of 2.5 L approx., using ethyl acetate (3 x 700 ml) in a 5 L separating funnel. Each time the separation funnel was shaken gently for a

few min and then left to settle until two distinct layers formed. The upper layers (ethyl acetate extract) were dried over sodium sulphate for 30 min. The ethyl acetate was then removed under reduced pressure using a rotary evaporator. Finally the crude yield of the metabolites was determined.

7.3 Chromatography process

Merck Kieselgel GF254 silica gel was used for column chromatography, thick layer and thin layer chromatography plates.

Pharmacia Fine Sephadex LH-20 was used in column chromatography to separate secondary metabolites from 6RD12 fungus (chapter 3) and Merck Kieselgel TLC silica gel 60 RP-18 $F_{254}S$. Column size and solvent systems used in each case are specified in context.

7.4 Spray reagents

- Anisaldehyde spray reagent was prepared as follows :

  Anisaldehyde (1.0 ml) and concentrated sulphuric acid (1.0 ml) were added to glacial acetic acid (98.0 ml).

- *p*-nitroaniline spray reagent was prepared as follows:

  A few drops of 5% sodium nitrite solution (see below) were added to 10% *p*-nitroaniline solution (50.0 ml) until the yellow colour of the solution disappears. Then sufficient quantity of 10% aqueous sodium carbonate solution was added until the yellow colour of the solution appears again.

  (The 10% solution of *p*-nitroaniline was prepared by dissolving 7.0 g of *p*-nitroaniline crystals in concentrated hydrochloric acid (90.0 ml) and then diluted to 1 L.

7.5 Crystallisation

The solvent conditions used for crystallisation of each pure secondary metabolite, with single or a mixture of solvents, is specified in each instance.

7.6 Physical properties

- Melting points were measured using a Kofler hot-stage apparatus and are uncorrected.
- IR spectra were obtained using a Perkin-Elmer Spectrum 100 FT-IR Spectrometer.
- ESI Mass spectra were determined using a Micromass ZMD (Single Quad) or a Micromass Quattro Ultima (Triple Quad).

  High resolution Mass Spectrometry analyses were carried out on a Thermo LTQ Orbitrap XL mass spectrometer.
- Optical rotations were measured using a Perkin Elmer 141 Polarimeter equipped with cell length 1.0 dm.
- ^{1}H and ^{13}C and 2D NMR spectra were obtained on a JEOL ECA 600 NMR spectrometer or a Bruker Avance-400 NMR spectrometer.
- Carbon atom types were determined by employing a combination of ^{13}C NMR broad-band proton-decoupled spectra, and Distortionless Enhancement by Polarisation Transfer (DEPT) experiments.
- Precise assignments were established by employing a combination of 1D and 2D NMR experiments.
- 2D NMR spectra were processed by JEOL Delta and/or ACD/Labs software.
- ^{1}H-^{1}H correlations by double quantum-filtered COSY.

- ^{1}H-^{13}C NMR correlations were determined by using HMQC, HMBC and H2BC pulse sequences.

7.7 Secondary metabolites from the fungus A311R

The fungus A311R was surface cultured on an aqueous solution of 3% malt extract with 6% added glucose. The fungus was firstly sub-cultured in 4 conical flasks (250 ml) for 3 weeks. It grew like white cotton on the surface of the medium. It started fruiting after 10 days. The initially white mycelium later turned brittle and black on the underside and supported un-branched, light brown-tipped xylaria-type fruiting bodies (Figure 97).

Figure 97 A311R fungus in a conical flask after 3 weeks

After 3 weeks, the fungus was transferred into 10×2 L Thompson bottles, each containing about 1000 ml of medium solution. After 8 weeks the mycelium was separated by filtration through a muslin cloth and left to air dry for 2 weeks. The brownish filtrate (10 L) was extracted in batches of 2.5 L in a separating funnel (5 L) with ethyl acetate (3 x 700 ml). The combined extracts were dried over anhydrous sodium sulphate for 30 minutes. After the removal of the ethyl acetate by rotary evaporation, a light brownish gummy mixture was obtained (4.32 g).

TLC examination of this crude extract using toluene, ethyl acetate and acetic acid (50:49:1) as eluent showed a brown spot at R_f 0.1 (R_f 0.3 in ethyl acetate : acetic acid 98:1) after spraying the plates with *p*-nitroaniline spray reagent.

7.7.1 Isolation of nonane-1,2,3-tricarboxylic acid (2.1) from A311R

The crude extract was dissolved in warm chloroform and set aside for 24 h. A light brownish solid (0.90 g) precipitated from the solution. The solid was recovered by filtration and crystallised from nitromethane to give nonane-1,2,3-tricarboxylic acid (**2.1**) as white yellowish glass-like crystals (375 mg), mp (117-120 °C); $HR_{(Orbitrap)}$ ESI $[M-H]^-$ *m/z* 259.1177, requires $C_{12}H_{20}O_6$; $[\alpha]_D^{20}$ - 186° (c 1.0, MeOH) ; $IR_{(ATR)}$ ν_{max} cm^{-1} : 3091, 1717, 1659 and 1175; ^1H NMR δ_H (C_5D_5N) 0.70 (3H, t, *J* 6.5 Hz, 10-CH_3), 1.10 (4H, m), 1.23 (2H, m), 1.47 (1H, m, 6a-H), 1.62 (1H, m, 6b-H), 1.86 (1H, m, 5a-H), 2.14 (1H, m, 5b-H), 3.46 (1H, m, 2-H) , 3.23 (1H, dd *J* 3.6, 14.0, 2-H), 3.50 (1H, m, 4-H) and 4.12 (1H, m, 3-H); ^{13}C NMR, δ_C (C_5D_5N) 14.04 (10-CH_3), 22.69 (9-CH_2), 28.32 (6-CH_2), 29.12 (5-CH_2), 29.49 (8-CH_2), 31.77 (7-CH_2), 33.94 (2-CH_2), 44.34 (3-CH), 47.42 (4-CH), 175.50 (11-COOH), 176.53 (1-COOH) and 177.00 (12-COOH).

7.7.2 Preparation of trimethyl nonane-1,2,3-tricarboxylate (2.1A)

A solution of **2.1** (50 mg) in methanol (1 ml) was reacted with ethereal diazomethane solution (2 ml). The resultant solution was stirred at 0 °C for 15 minutes. Then the solvent was removed by rotary evaporation to yield a yellowish-brown oily material. The latter was triturated with warm petroleum ether (60-80 °C) to give trimethyl nonane-1,2,3-tricarboxylate (**2.1A**) as a brown oil (23 mg); ES $[M+H]^+$ *m/z* 303 ($C_{15}H_{26}O_6$); $IR_{(ATR)}$ ν_{max} cm^{-1} : 1735, 1743 and 1240; ^1H NMR, δ_H (C_5D_5N) 0.72

J 7.0 Hz, 10-H), 1.08 (6H, m), 1.23 (2H, m), 1.49 (1H, m, 5a-H), 1.73 (1H, m, 5b-H), 2.77 (1H, dd, *J* 4.3, 12.5, 2-H), 2.92 (2H, m, 2a, 4-H), 3.43 (1H, m, 3-H), 3.52 (3H, s, 11-COO*Me*), 3.59 (3H, s, 12-COO*Me*), 3.61 (3H, s, 1-COO*Me*); ^{13}C NMR, δ_C (C_5D_5N) 13.98 (10-CH_3), 22.59 (9-CH_2), 27.46 (6-CH_2), 29.10 (5-CH_2), 31.56 (8-CH_2), 29.13 (7-CH_2), 33.26 (2-CH_2), 43.24 (3-CH), 46.52 (4-CH), 51.58 (14-O*Me*), 51.65 (15-O*Me*), 51.92 (13-O*Me*) ,172.28 (11-*C*), 174.25 (1-*C*) and 173.50 (12-*C*).

7.7.3 Preparation of ethereal alcoholic solutions of diazomethane [1-2]:

Ethanol (25ml, 95%) was added to a solution of potassium hydroxide (5 g) in water (8 ml) in a (100 ml) distillation flask fitted with dropping funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series, the second of which contained ether (25 ml). The inlet tube of the second receiver dipped below the surface of the ether, and both receivers were cooled to 0 °C. The flask containing the alkali solution was heated in a water bath to 65 °C, and a solution of diazald (21.5 g, 0.1 mole) in about 200 ml of ether was added through the dropping funnel in about 25 minutes. The rate of distillation should be about equal the rate of addition. When the dropping funnel was empty, another 40 ml of ether was added slowly and the distillation was continued until the distilling ether was colourless .The combined ethereal distillate contained about 3 g of diazomethane.

Diazomethane is not only exceedingly toxic, but its solutions have been known to explode quite unexpectedly. Hence, all work with diazomethane, regardless of how it is generated, should be carried out behind safety shields in efficient hoods.

7.7.4 Isolation of spiculisporic acid (2.2) from A311R

A brown spot was observed on a silica gel plate at R_f 0.3 developed using ethyl acetate : acetic acid (99:1) mobile phase, when sprayed with *p*-nitroaniline spray reagent. 1 g of the ethyl acetate crude extract was applied to preparative silica plates (5 × 20 cm × 20 cm) to isolate this compound. Ethyl acetate : acetic acid (99:1) was used as mobile phase to develop the plate. The band was then removed and washed with ethyl acetate to give light brown solid, which was crystallised from nitromethane to give colour less crystals of spiculisporic acid (28 mg); mp 142-145 °C, (lit.[3], mp 140-143 °C); $HR_{(Orbitrap)}$ ESI $[M-H]^-$ *m/z* 327.1809 ($C_{17}H_{28}O_6$); $[\alpha]_D^{20}$ - 12° (c 0.25, EtOH), (lit. [3] , $[\alpha]_D^{20}$ – 15° (EtOH); $IR_{(ATR)}$ ν_{max} cm^{-1} : 2952, 2551, 1791, 1778 and 1710; ^1H NMR, δ_H (C_5D_5N) 0.77 (3H, td, *J* 6.0, 2.3 Hz, 15-CH_3), 1.07 (8H, m), 1.14 (4H, m), 1.53 (1H, m, 7a-H), 1.68 (1H, m, 7b-H), 1.93 (1H, m, 6a-H), 2.33 (1H, m, 6b-H), 2.70 (1H, m, 3b-H), 2.83 (3H, m, 2a, 2b and 3a-H), 3.66 (1H, dd *J* 10.77, 2.75, 5-H); ^{13}C NMR, δ_C (C_5D_5N) 14.14 (15-CH_3), 22.76 (14-CH_2), 28.48 (7-CH_2), 28.74 (6-CH_2), 28.75 (2-CH_2), 29.39 (8-CH_2), 29.57 (12-CH_2), 29.68 (11-CH_2), 29.69 (9,10-CH_2), 30.12 (13-CH_2), 31.92 (3-CH_2), 52.14 (5-CH), 87.63 (4-C), 174.52 (16-COOH), 175.12 (17-COOH) and 176.82 (1-C).

7.7.5 Preparation of Dimethyl ester of spiculisporic acid

A solution of spiculisporic acid (15 mg) in methanol (1 ml) was reacted with ethereal diazomethane solution (2 ml). The resultant solution was stirred at 0 °C for 15 minutes. Then the solvent was evaporated to yield a yellowish-brown oily material. The latter was triturated with hot petroleum ether (60 -80 °C) to give dimethyl spiculisporate as a brown waxy material (7 mg); $HR_{(Orbitrap)}$ ESI $[M - CH_3]^-$ *m/z* 341.1966 ($C_{19}H_{32}O_6$); $IR_{(ATR)}$ ν_{max} cm^{-1} : 2923, 1764 and 1740; ^1H NMR, δ_H

0.78 (3H, td, *J* 6.0, 2.3 Hz, 15-H), 1.11 (11H, m), 1.18 (4H, m), 1.26 (1H, m, 7a-H), 1.34 (1H, m, 6b-H), 1.65 (1H, m, 6a-H), 2.56 (3H, m, 12a and 12b, 4-H), 2.67 (1H, m, 3a-H), 3.26 (1H, dd, J 10.77, 2.75, 5-H), 3.67 (3H, s, 17-COO*Me*), 3.63 (3H, s, 19-COO*Me*); ^{13}C NMR, δ_C (C_5D_5N) 14.13 (15-CH_3), 22.77 (14-CH_2), 27.92 (7-CH_2), 27.95 (6-CH_2), 27.99 (2-CH_2), 29.37 (8-CH_2), 29.40 (12-CH_2), 29.43 (11-CH_2), 29.56 (9-CH_2), 29.64 (10 and 13-CH_2), 31.94 (3-CH_2), 52.88 (5-CH), 86.52 (4-C), 51.85 (18-OMe), 51.88 (19-O*Me*), 171.53 (16-*C*), 172.54 (17-*C*) and 175.68 (1-*C*).

7.8 Secondary metabolites from the fungus 6RD12

The fungus 6RD12 was collected in Thailand and classified as a *xylaria* species. The front view of the Petri dish showed a white mycelium overlaid with some black concentric rings. The back view of the Petri dish of the fungus 6RD12 showed an orange underside to the mycelium (Figure 98).

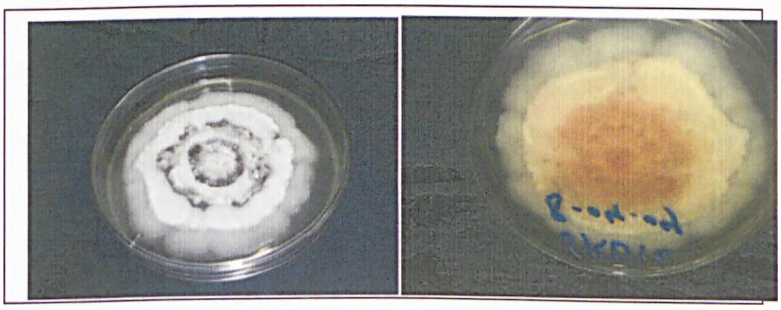


Figure 98 Front and back view of 6RD12 in the Petri dish

The fungus 6RD12 was firstly surface sub-cultured on an aqueous solution of 3% malt extract with 6% added glucose.in 4 conical flasks (250 ml) for 3 weeks. Initially a white cotton wool like mycelium formed on the medium surface. It started fruiting after 10 days. It developed black cylindrical stromata with white tips.

The fungus was then cultured in Thompson bottles (8 × 2 L) for 8 weeks. The fungus during the first 4 weeks produced a white mycelium, which then developed black areas.

After 8 weeks the mycelium was separated by filtration through a muslin cloth and left to air dry for 2 weeks. The brownish filtrate (8 L) was extracted in batches of 2.5 litres approx. with ethyl acetate (3 x 700 ml) using a separating funnel (5 L). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate for 30 minutes. After the removal of the ethyl acetate by a rotary evaporator, a light brownish gummy mixture was obtained (3.35 g).

7.8.1 Isolation and structure elucidation of 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one 3.1

A normal phase TLC of the ethyl acetate extract eluted with toluene, ethyl acetate and acetic acid (50:49:1) revealed little of interest other than a dark brown spot at the origin of the TLC plate. As a result, a reverse phase TLC plate was used with methanol : water (50:50) as eluent. The plate showed 3 UV active spots at R_f 0.96, 0.85 and 0.46. A chromatography column (50 × 2.0 cm) was packed with Sephadex LH20 (70 g) in methanol-water (80:20). The crude extract (3 g) was adsorbed onto the top of the column, which was eluted with a methanol-water (80:20). Fractions of 4 ml were collected. The first compound (**3.1**) collected from (tubes 24-26, methanol-water (80:20), was 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one as a white solid (95 mg), mp 180-185 °C, $HR_{(Orbitrap)}$ ESI $[M+H]^+$ *m/z* 243.12274, ($C_{12}H_{18}O_5$); $[\alpha]_D^{22}$ - 160° (c 0.5, MeOH); $IR_{(KBr)}$ ν_{max} cm^{-1}: 3378, 1673, 1105 and 1402; ^1H NMR (C_5D_5N) 0.82 (3H, t, *J* 7.4 Hz, 13-H), 1.43(m, 12b-H), 1.62 (2H, m, 11b-H and 12a-H), 2.04 (1H, m, 11a-H), 3.65 (1H, td, *J* 2.75,

8.1 Hz , 3-H), 4.75 (1H, m, 4-H), 5.42 (1H, bs, 5-H), 4.51 (1H, m, 6-H), 2.81 (1H, dd, *J* 3.6, 15.81 Hz 7a-H), 3.21 (1H, dd, *J* 8.1, 15.81, 7b-H), 4.77 (1H, d, *J* 15.81, 1a-H), 4.42 (1H, dt, *J* 2.4, 16.3, 1b-H); ^{13}C NMR, δ_C (C_5D_5N), 14.17 (13-CH_3), 18.98 (12-CH_2), 34.91 (11-CH_2), 79.08 (3-CH), 66.82 (4-CH), 66.48 (5-CH), 66.59 (6-CH), 43.50 (7-CH_2), 63.76 (1-CH_2), 132.6 (9-C), 155.27 (10-C), 197.08 (8-C).

7.8.2 Isolation and structure elucidation of cycloepoxydon 3.2 from fungus 6RD12

The fractions (38-44, methanol-water 80:20) were collected and combined together according to the Rp TLC plate where they showed weak UV absorption at R_f 0.85. After the solvent was removed, the residual brownish white solid (17.4 mg) was recrystallised from nitromethane to give cycloepoxydon as colourless crystals (7.3 mg) , mp 186-189 °C, ES $[M+H]^+$ *m/z* 241; $[\alpha]_D^{22}$ - 139° (c 0.25, $CHCl_3$:MeOH (95:5), {lit. [4] $[\alpha]_D^{22}$ - 145° [c 1.1, $CHCl_3$:MeOH (95:5)] [4]; $IR_{(KBr)}$ ν_{max} cm^{-1}: 3378, 1676, and 1105 ; ^1H NMR [$(CD_3)_2CO$], δ_H 0.89 (3H, t, *J* 7.2 Hz, 13-H), 1.36 (1H, m, 12a-H), 1.38 (1H, m, 11a-H), 1.41(1H, m, 12b-H), 1.79 (1H, m, 11b-H), 3.19 (td, *J* 2.6, 8.4, Hz , 3-H), 3.34 (1H, d, *J* 4.1 Hz, 7-H), 3.85 (1H, t, *J* 3.95, 6-H), 4.12 (1H, dd, 16.9, 1.9 Hz, 1aH), 4.15 (2H, m, 1b-H and 4-H), 4.95 (1H, bs, 5-H); ^{13}C NMR, δ_C [$(CD_3)_2CO$], 13.52 (13-CH_3), 18.49 (12-CH_2), 34.4 (11-CH_2), 78.27 (3-CH), 66.76 (4-CH), 63.63 (5-CH), 55.12 (6-CH), 53.21 (7-CH), 63.29 (1-CH_2), 128.49 (9-C), 151.79 (10-C), 192.73 (8-C).

7.9 Secondary metabolites from the fungi A217R and A517R

The Petri dishes front and back views of A217R and A517R looked similar. Both fungi were sub-cultured in conical flasks of aqueous 3% malt to prepare them for

large scale culturing. After 3 weeks the conical flask content were transferred into Thomson bottles (6 × 2 L) containing aqueous 3% malt enriched with 6% glucose (1 L) and left to grow for eight weeks. They formed a white gelatinous mycelium (Figure 99). The mycelium was removed by filtration through muslin cloth and the aqueous filtrate extracted in portions with ethyl acetate (3 x 700 ml) in a separating funnel (5 L). The combined extracts were dried over sodium sulphate for 30 min. and the ethyl acetate removed by a rotary evaporator to give a brownish gummy material (5.5 g of A217R and 6.2 g of A517R).

Figure 99 Fungus A517R in Thomson bottle after 8 weeks

The extracts were then analyzed for their content by hand made TLC plates made from Merck Kieselgel GF254 silica gel. A solvent system of toluene: ethyl acetate: acetic acid (50:49:1) was used to develop the plates. Both fungi showed similar TLC results. Under visible light, a violet coloured spot was observed at R_f. of 0.58. Under short wave UV (245 nm), three UV active spots were detected; a weaker spot at the lower R_f. of 0.27 and two stronger spots at 0.65 and 0.90.

TLC plate sprayed with *p*-nitroaniline developed a strong orange colour spots at R_f 0.65 and 0.90 and an opaque spot at R_f. of 0.27.

7.9.1 Isolation and structure elucidation of cytochalasin D from A217R

The ethyl acetate extract (5.5 g) was triturated with ethyl acetate and left overnight. A white precipitate was obtained. The white solid was filtered off and recrystallized from ethyl acetate to give cytochalasin D as white needle crystals (65 mg), mp 238-240 °C; ES [M+H]+ *m/z* 508 (consistent with $C_{30}H_{37}NO_6$); $IR_{(KBr)}$ ν_{max} cm^{-1}: 3681, 2919, 1739 and 1689; ^1H NMR (C_5D_5N) δH 0.77 (3H, d, *J* 6.70 Hz, 11-H), 0.98 (3H, d, *J* 6.50 Hz, 22-H), 1.50 (3H, s, 23-H), 1.93 (1H, dd, *J* 5.0, 11.8 Hz, H-15), 2.33 (3H, s, 25-H), 2.42 (1H, m, 4-H), 2.66 (1H, m, 16-H), 2.86 (2H, m, 10-H), 2.97 (1H, m, 5-H) 3.38 (1H, t, *J* 10.5, 8-H), 3.55 (1H, m, 3-H), 4.39 (1H, d, *J* 10.1 Hz, 7-H), 5.41 (3H, s, 12-H), 5.60 (1H, m, 13-H), 5.57 (1H, m, 19-H), 6.01 (1H, s, 21-H), 6.34 (2H, m, 14-H), 6.84 (1H, dd, *J* 2.23, 15.8 Hz, H-20), 7.14 (2H, m, Ar-H), 7.22 (2H, m, Ar-H), 7.24 (2H, m, Ar-H); ^{13}C NMR(C5D5N) δc 13.59 (11-CH3), 19.34 (22-CH3), 20.51 (25-CH3), 24.61 (23-CH3), 33.14 (5-CH), 38.54 (15-CH2), 42.43 (16-CH), 45.49 (10-CH2), 47.87 (8-CH), 50.07 (4-CH), 53.99 (9-C), 54.36 (3-CH), 71.27 (7-CH), 77.99 (21CH), 78.39 (18-C), 112.17 (12 =CH2), 126.79 (13-CH), 127.84 (29-Ar-CH), 128.79 (28, 30-ArCH), 129.90 (27, 31-ArCH), 132.95 (20-CH), 132.80 (14-CH), 133.82 (19-CH), 138.44 (26-ArCH), 151.63 (6-C=), 170.47 (24-C=O), 175.11 (1-C=O) and 210.88 (17-C=O).

7.9.2 Isolation and structure elucidation of S-mellein from A217R

After the isolation of cytochalasin D, the ethyl acetate crude extract obtained from the fungus A217R (4.0 g) was treated with warm toluene to give a yellow oily material (1.1 g). Preparative TLC (8x, 20 × 20 cm) was run using toluene : ethyl acetate : acetic acid (50:49:1) solvent system to separate the content of the toluene fraction, which showed two main compounds at R_f 0.90 and 0.65. The UV

fluorescent band at R_f 0.90 was recovered and then washed with ethyl acetate to produce a clear yellow solution. The ethyl acetate was removed under low pressure to yield yellow oil (34 mg). The oil was triturated with *n*-hexane to produce yellow solid (7.5 mg), which was recrystallised from the same solvent to give S-mellein as white plates (4.0 mg). mp 53-55 °C; ES $[M+H]^+$ *m/z* 179; $[\alpha]_D^{23}$ + 85 (c 0.05, MeOH), [lit [5]. mp 55-56 (ether/hexane), $[\alpha]_D^{25}$ +88.6 (c 0.27, MeOH]; ^{1}H NMR: (600 MHz, $CDCl_3$) δ_H 1.54 (d, *J* 6.27 Hz, 3 H, 9-H) 2.96 (d, *J* 7.13 Hz, 2 H, 4-H) 4.74 (m, 1 H, 3-H) 6.72 (d, *J* 7.39 Hz, 1 H, 5-H) 6.91 (d, *J* 8.51 Hz, 1 H, 7-H) 7.44 (dd, *J* 8.42, 7.39 Hz, 1 H, 6-H) 11.01 (s, 1 H, 8-OH). ^{13}C NMR ($CDCl_3$) δ_C 20.80 (CH_3, 11-C), 34.62 (CH_2, 4-C) 76.15 (CH, 3-C), 108.29 (C, 9-C), 116.23 (CH, 7-C), 117.95 (CH, 5-C), 136.19 (CH, 6-C), 139.43 (C, 4-C), 162.17 (C-OH, 8-C), 170.00 (C=O, 1-C).

7.9.3 Isolation and structure elucidation of *cis*-4-Hydroxymellein from A217R

The next UV active band in the PTLC at R_f 0.64 was removed and then washed with ethyl acetate to produce a clear yellow solution. The ethyl acetate was removed under low pressure to produce yellowish needles (255 mg) which were recrystallized from toluene to give *cis*-4-hydroxymellein as white needles (119 mg), mp 117-120 °C; ES $[M+H]^+$ *m/z* 195, $[\alpha]_D^{23}$ + 38° (c 0.5, MeOH), [lit. [6], mp 118-119 °C, $[\alpha]_D^{20}$ + 37.4° (c 033, MeOH)]; $IR_{(KBr)}$ ν_{max} cm^{-1} 3427, 3178, 2997, 1675; ^{1}H NMR: (600 MHz, $CDCl_3$) δ_H 1.58 (3H, d, *J* 6.6 Hz, 9-H), 4.55 (1 H, d, *J* 2.10 Hz, 4-H) 4.68 (1 H, qd, *J* 2.10, 6.62 Hz, 3-H) 6.91 (1 H, d, *J* 7.30, Hz, 5-H) 7.01 (1 H, d, *J* 8.51 Hz, 7-H) 7.51 (1 H, dd, *J* 8.51, 7.30 Hz, 6-H) 10.97 (1 H, s, 8-OH). ^{13}C NMR: ($CDCl_3$) δ_C 16.15 (CH_3, 11-C), 67.37 (CH, 3-C), 78.33 (CH-OH, 4-C), 106.93 (C, 9-C), 118.44

(CH, 7-C), 118.67 (CH, 5-C), 136.94 (CH, 6-C), 140.60 (C, C-10), 162.20 (C, 8-C), 169.33 (C=O, 1-C).

7.9.4 Secondary metabolites from A517R

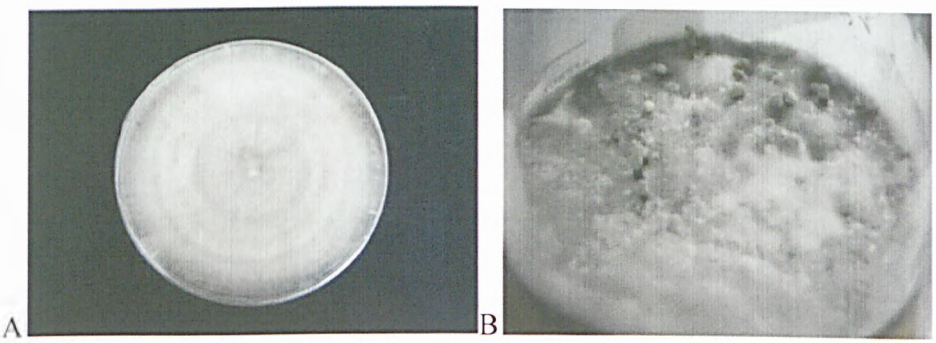
The same procedures described for the isolation of secondary metabolites of A217R were applied to the ethyl acetate crude extract of the fungus A517R. It produced cytochalasin D (54 mg), mellein (5 mg) and *cis*-4-hydroxymellein (72 mg).

It was found that this fungus produced exactly the same secondary metabolites as A217R, which suggests that the two fungi are possibly the same species.

The mycelia of A217R and A517R did not show any interesting secondary metabolites.

7.10 Secondary metabolites from fungus X04

The fungus X04 was surface cultured on an aqueous solution of 3% malt enhanced with 6% glucose. The fungus was firstly sub-cultured in 5 conical flasks (250 ml) for 2 weeks. It grew like white cotton on the medium surface. It was fruiting after 12 days (Figure 100).



A) in a Petri dish **B) in a conical flask after 2 weeks.**

Figure 100 Fungus X04

After 2 weeks, the fungus was transferred into 30 × 1 L Thompson bottles, each containing about 500 ml of medium solution. Figure 101 shows fungus X04 inside the Thompson bottle after 4 weeks.

After 8 weeks the mycelium was separated by filtration through a muslin cloth and left to air dry for 10 days. The brownish filtrate (15 L) was extracted in portions of 2.5 L with ethyl acetate (700 ml) three times. The combined extracts were dried over anhydrous sodium sulphate for 30 minutes. After the recovery of ethyl acetate by a rotary evaporator, a light brownish gummy mixture was obtained (8.5 g).

Figure 101 Fungus X04 inside the Thompson bottle after 4 weeks.

TLC examination of this crude extract in different solvents system showed that this extract was a mixture of several minor compounds but only two major components.

7.10.1 Isolation of Coriloxin from fungus X04

The gummy mixture was triturated with hot toluene and left overnight. A yellow solid (4.31 g) was obtained, which was recrystallized 3 times from toluene to give coriloxin as colourless needle-like crystals (2.5 g), mp 153-155 °C (lit. [7] mp 153-155) ; ES $[M+H]^+$ *m/z* 171 (consistent with $C_8H_{10}O_4$); $[\alpha]_D^{23}$ -98.4° (c 0.5, MeOH),

[lit., [7] $[\alpha]_D^{20}$ -100° (c 033, EtOH]; $IR_{(KBr)}$ ν_{max} cm^{-1} 3398, 2942, 1650, 1606; ^1H NMR ($CDCl_3$) δ_H 5.26 (1H, d, 2.02 Hz), 4.49 (1H, d, 4.55 Hz), 3.77 (3H, s), 3.34 (1H, d, 2.02 Hz), 2.69 (1H, d, 4.55 Hz) and 1.66 (3H, s); ^{13}C NMR; δ_C ($CDCl_3$) 18.91 (8-CH_3), 56.56 (7-CH_3), 59.43 (5-C), 60.55 (6-CH), 69.10 (4-CH), 98.18 (2-CH), 171.40 (3-C), 193.41 (1-C);

7.10.2 Isolation of S-mellein from fungus X04

The ethyl acetate extract of the medium of X04 (4.3 g) was chromatographed over a silica gel column (60 × 2.5 cm). The column was eluted with a mixture solvent system petroleum ether, ethyl acetate and acetic acid (50:50:1) and fractions of 3.0 ml were collected using a fraction collector.

The first component recovered (tubes 74-83) was yellowish oil(R_f 0.92), which was triturated with *n*-hexane to give a white solid (8 mg), which was recrystallised from the same solvent to give S-mellein as white plates (3 mg) , mp 52-55 °C; ES $[M+H]^+$ *m/z* 179; $[\alpha]_D^{23}$ -117° (c 0.05, MeOH), (lit. [8] $[\alpha]_D^{22}$ -100° (c 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ_H 1.51 (3H, d, *J* 6.42 Hz), 2.91 (2H, d, *J* 6.88 Hz), 4.71 (1H, m), 6.67 (1H, d, *J* 7.34 Hz), 6.87 (1H, d, *J* 8.25 Hz), 7.39 (1H, dd, *J* 7.39 and 8.42 Hz), 11.01 (1H, s); ^{13}C ($CDCl_3$) δ_C 20.88 (11-CH_3), 34.70 (4-CH_2), 76.24 (3-CH), 116.32 (7-CH), 118.04 (5-CH), 136.27 (6-CH), 108.38 (9-C), 139.52 (10-C), 162.27 (8-C), and 170.09 (1-C).

7.10.3 Isolation of *cis,trans*-4-hydroxymellein mixture from fungus X04

The second component (tubes 38-52) collected from the column gave a white solid (12 mg), which was recrystallised from petroleum ether (60-80 °C) as fine white needles (4.3 mg) and found to be a mixture of two 4-hydroxymellein stereoisomers

($C_{10}H_{10}O_4$); mp 117-122 °C; ES $[M+H]^+$ *m/z* 195; ^{1}H NMR, δ_H ($CDCl_3$) 1.50 (3H, d, *J* 6.19 Hz, CH_3), 1.56 (3H, d, *J* 6.59 Hz, CH_3), 2.15 (1H, s(br), OH), 4.68 (1H, dq, *J* 2.06 and 6.70, Hz, 4-H), 4.60 (1H, m), 4.56 (1H, m), 4.58 (1H, m), 6.91(1H, d, *J* 7.22 Hz, Ar-H), 6.97(1H, d, *J* 8.43 Hz, Ar -H), 7.00 (1H, dd, *J* 8.43 Hz, Ar -H), 7.01 (1H, d, *J* 7.70 Hz, Ar -H), 7.51 (1H, dd, *J* 8.43, 7.70 Hz, Ar -H), 10.99 (1H, s, chelated OH); ^{13}C NMR, δ_C ($CDCl_3$) 16.09, 18.00 (11-CH_3); 67.29, 69.19 (3-CH); 78.30, 80.06 (4-CH); 106.73, 106.91 (9-C); 116.35, 117.87 (7-CH); 118.39, 118.60 (5-CH); 136.89, 136.97 (6-CH); 140.58, 141.27 (10-C); 162.05, 162.14 (8-C); 168.59, 169.29 (1-C).

7.10.4 Isolation of 2-hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione from fungus X04

The third component from the column chromatography was a mixture of two compounds (tubes 110-170). Three 20 × 20 cm PLC plates were used to separate 300 mg of the mixture. The chromatogram was developed using toluene, ethyl acetate and acetic acid (50:49:1) as the solvent system. The violet band was removed and the compound was extracted from the silica by washing with ethyl acetate to give orange crystals after evaporation of the solvent. Recrystallisation from petroleum ether (bp 80-100 °C) gave orange crystals of 2-hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione (8.7 mg), mp 117-122 ° C. ES $[M+H]^+$ *m/z* 183 (consistent with $C_9H_{10}O_4$), $IR_{(KBr)}$ ν_{max} cm^{-1}, 3348, 2925, 1640, 1598 and 1460; ^{1}H NMR ($CDCl_3$), δ_H 1.49 (3H, t, CH_3), 1.93 (3H, s), 4.03 (2H, q, CH_2), 5.79 (1H, s) and 7.28 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ_C, 7.98 (CH_3), 13.92 (CH_3), 65.96 (CH_2O), 102.51 (CH), 114.88 (C-3), 151.61 (C-2), 160.57, (C-5), 182.22 (C=O), 182.85 (C=O).

7.11 Secondary metabolites from fungus 6RD8

The fungus 6RD8 (Figure 102) was collected from Thailand and classified as a *xylaria* species. It was cultured on an aqueous solution of a mixture of 3% malt extract and 6% glucose for 8 weeks. It yielded a thin yellowish white mycelium and 4 L brown medium.

Figure 102 6RD8 fungus

7.11.1 Isolation of 8-*O*-methylmellein from fungus 6RD8

Ethyl acetate extraction of the culture medium gave light brown gum (1.7 g). Trituration of the gum with light petroleum ether (60-80 °C) afforded white cubic crystals, which were recrystallized from the same solvent to give S-8-O-methylmellein (65.5 mg), mp 84-87 °C (lit. [5]. 86.5-87.5, ether/hexane); ES $[M+H]^+$ *m/z* 193 ($C_{11}H_{10}O_3$); $[\alpha]_D^{22}$ + 210 ° (c 1.0, MeOH) [lit. [9] $[\alpha]_D^{23}$ + 238 ° (c 1.02, $CHCl_3$)]; $IR_{(ATR)}$ ν_{max} cm^{-1} 2918, 1709, 1596; ^1H NMR ($CDCl_3$), δ_H 1.45 (3H, d, *J* 6.42 Hz, 11-CH_3), 2.84 (2H, m, 4-CH_2), 3.92 (3H, s, 12-CH_3O), 4.53 (1H, m, 3-H), 6.76 (1H, d, *J* 7.34 Hz, 7-H), 6.89 (1H, d, *J* 8.25 Hz, 5-H), 7.43 (1H, dd, *J* 7.57, 8.43 Hz, 6-H); ^{13}C NMR, δ_C ($CDCl_3$) 20.79 (11-CH_3), 36.19 (4-CH_2), 56.27 (12-CH_3), 74.22 (3-CH), 110.98 (5-CH), 113.74 (9-C), 119.29 (7-CH), 134.55 (6-CH), 142.08 (10-C), 161.27 (8-C) and 162.86 (1-C).

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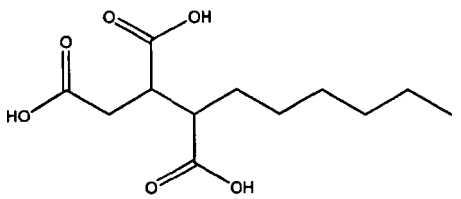
Chapter 8 Summary

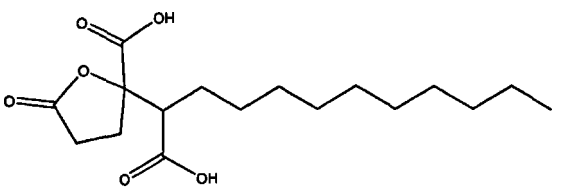
Endophytic fungi are an important source of novel compounds [1] and bioactive agents, which can lead to the development of new drugs for the pharmaceutical and agricultural industries [2].

In this study, secondary metabolites from six endophytic *Xylaria* fungi, collected from the Kao Loung National Park in Thailand, were investigated. The structures of the compounds were elucidated by chemical and spectroscopic methods.

The fungi were static surface cultured on an aqueous malt extract-glucose medium. The fungus A311R produced nonane-1,2,3-tricarboxylic acid (Figure 103), which was isolated for the first time as a natural product and its trimethyl ester derivative was prepared. Also isolated from the same fungus was spiculisporic acid (Figure 103); the first instance of isolation from a *Xylaria* fungus. The dimethyl ester of spiculisporic acid was also prepared.



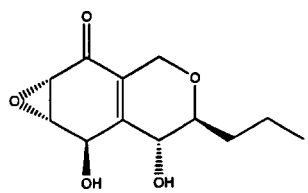
nonane-1,2,3-tricarboxylic acid



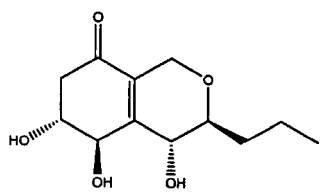
spiculisporic acid

Figure 103 Secondary metabolite isolated from the fungus A311R

The fungus 6RD12 produced; cycloepoxydon a known compound, which has been isolated for the first time from a *Xylaria* fungus, and 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one, which is a novel compound (Figure 104).



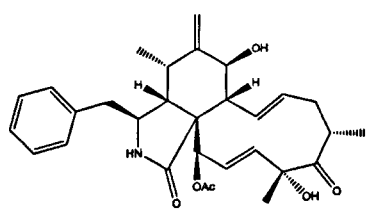
cycloepoxydon



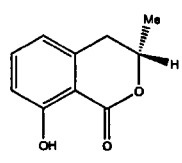
4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one

Figure 104 Secondary metabolites isolated from the fungus 6RD12.

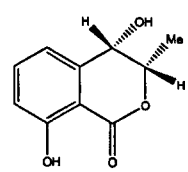
The fungi A217R and A517R produced cytochalasin D, (S)-mellein and (3S,4S)-4-hydroxymellein as main secondary metabolites (Figure 105) suggesting that the two fungi are the same species.



cytochalasin D



(S)-mellein

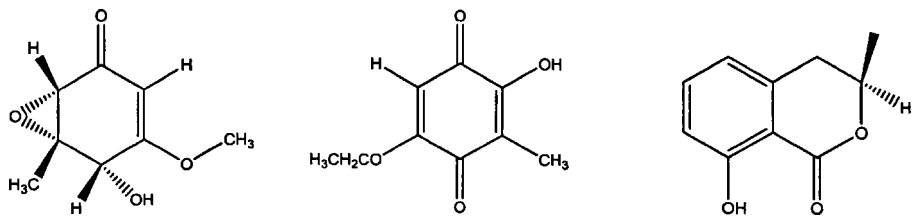


4-hydroxymellein

Figure 105 Secondary metabolites isolated from fungi A217R and A517R

The fungus X04 (*Xylaria* cf. *juruensis*) produced 2-hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione as a novel compound, coriloxin as the main

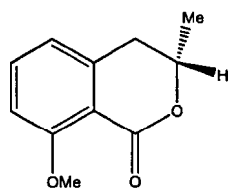
secondary metabolite in addition to (R)-mellein (Figure 106) and a mixture of two isomers of the 4-hydroxymellein.



coriloxin | 2-hydroxy-5-ethoxy-3-methylcyclohexa-2,5-dien-1,4-dione | (R)-mellein

Figure 106 Secondary metabolites isolated from X04 and 6RD8 fungi

The fungus 6RD8 produced (S)-*O*-methylmellein as the main secondary metabolite (Figure 107).



(S)-*O*-methylmellein

Figure 107 Secondary metabolite isolated from 6RD8 fungus

4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one, Coriloxin, cytochalasin D, mellein and 4-hydroxymellein were tested as anti-plasmodial agent against Plasmodium falciparum strain K1. 4,5,6-trihydroxy-3-propyl-3,4,6,7-tetrahydro-1*H*-isochromen-8(5*H*)-one gave IC_{50} = 3.74 μg/mL (mean of two experiments). Coriloxin gave IC50 = 2.22 μg/mL (mean of two experiments), whilst the other compounds showed no activity at the highest dose tested (50 μg/mL).

References

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